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EFFECTS OF NITROGEN ON CHLOROPHYLL, ACIDITY, ASCORBIC ACID, AND CARBOHYDRATE FRACTIONS OF *ANANAS COMOSUS* (L.) MERR.[1]

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(WITH EIGHT FIGURES)

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Introduction

Well-authenticated information on pineapple plant response to nitrogen constitutes a valuable criterion in commercial fertilizer practices because the amounts and time of their application are determined by the physical appearance of the plants, designated as plant status, as well as by chemical analyses of their tissues.

Besides nutrition, physical factors such as light, temperature, and moisture may also influence the appearance or status of plants as measured by the chlorophyll content, succulence, and texture of the leaves. Under greenhouse conditions light was approximately 30% lower and temperature from three to five degrees higher than in the open.

Data presented in a previous publication (**26**) showed that growth and intake of nutrient mineral elements by *A. comosus* grown in nutrient solutions under greenhouse conditions increased more with high than with low amounts of nitrogen. Data for the same plants on dry matter, chlorophyll, titrable acidity, ascorbic acid, and various carbohydrate fractions are presented below.

Methods

Cultural methods were presented in a previous publication (**26**). The morphological basis of leaf nomenclature, the technique for sectioning the various organs, and the preparation of the tissues for chemical analysis have also been reported (**22**).

A brief review of the system of leaf nomenclature, originally reported elsewhere (**22**), may familiarize the reader with the different groups of leaves

[1] Published with the approval of the Director as Technical Paper no. 168 of the Pineapple Research Institute, University of Hawaii.

and the position of their sections. Leaves having completed growth at the time of planting are designated as senile (A), and those having attained only partial growth as old (B). The leaves which are formed after planting are grouped according to their chronological age and physical appearance at the time of plant harvesting as mature (C), active (D), or young (E). All leaves are cut to four or five sections which may differ in physical appearance and chemical composition. The basal section, which is composed of meristematic tissues in leaves not having attained complete development and lacking in chlorophyll, is designated as no. 1, and the immediately adjoining section, which is partly chlorophyllous and occupies the position between the non-chlorophyllous and chlorophyllous sections, as no. 2. Section nos. 3, 4, and 5 are chlorophyllous but differ in chemical composition, presumably because of differences in chronological age and rates in physiological functioning.

Synoptic expressions have been introduced for the often repeated, long appellations of the cultures and series. Thus the cultures with 140.0 mg. of nitrogen per liter of solution are designated as high-N and those with 2.8 mg. as low-N cultures.

Review of literature

The literature on carbohydrate and nitrogen relations is very extensive. It is mainly concerned with the effects of varying amounts of nitrogen on carbohydrate accumulations in plant tissues and the interdependence of vegetative growth or fructification of such carbohydrate and nitrogen relations.

NIGHTINGALE'S studies (**18**) on the nitrate and carbohydrate reserves in relation to the nitrogen nutrition of pineapples indicated that: "It is essential to maintain an adequate reserve of nitrate in the plant. If the concentration of nitrate is relatively low . . . its reduction is not so efficiently or freely effected as when it is higher. Sufficient carbohydrates must be available for oxidation as nitrate is reduced." In a later publication (**19**) he reported: "In the pineapple plant a low reserve of nitrate was found adequate for greatest possible yield of fruit when carbohydrates were low, in contrast with the requirements of the plants of high nitrate reserves for maximum production when carbohydrates were high." WADLEIGH (**29**) observed in cotton plants that hemicellulose reserves had not been affected at all, while sugars and starch were more abundant in the tissues of the low than high nitrogen cultures, but the yield of bolls was 5.3 times greater in the latter than former cultures. The studies of RUSSELL and BISHOP (**21**) on the relationships of nitrogen and carbohydrates in barley showed that: "Nitrate assimilation is normally most active in the early part of the plant's life and carbohydrate synthesis continues much later but its total amount depends on the quantity of nitrate taken up so that the two processes, though quite distinct, are nevertheless closely related." MURNEEK (**15**) observed that flowering of apples is characterized by marked increase of all active forms of carbohydrates and nitrogen, while leaf and fruit development is

characterized by an accumulation of nitrogen in water insoluble form which is plentiful in the leaves early in the season; later the nitrogen distribution is reversed. TRAUB (**28**) noted that apple twigs contained, during the most active growth period, higher amounts of amino nitrogen, but lower amounts of protein nitrogen, total sugars, and total carbohydrates. DAS (**3**) found in sugar cane that increasing nitrogen supplies increased the size of the leaf, the rate of leaf and joint formation, rate of elongation, tillering, yield of cane, uptake of ash constituents, soluble nitrogenous fractions, succulence, and content of reducing sugars, but decreased the concentration of sucrose in the expressed juice. MACY (**10**) expounded a theory called "the relationship between sufficiency of a nutrient and its percentage content in the plant" which claimed to show that maximal yields of barley and straw are obtained when a certain critical percentage of nitrogen in the tissues is attained. MACY's theory may be challenged by BOYNTON and COMPTON's (**1**) statement that chemical analysis of leaves for nitrogen, potassium, or magnesium cannot take the place of careful observations on tree behavior, appearance, and development of visible leaf or fruit symptoms; but that such analysis coupled with these observations may make possible a positive diagnosis that neither alone would have permitted. LOEHWING (**9**) observed in oats, corn, and wheat that high yield in young plants was associated with high carbohydrates and high organic nitrogen, and low yield, with low protein, low carbohydrate and high nitrate content. According to GAUCH and EATON (**4**), carbohydrate accumulations in barley plants grown in solutions with low and high concentrations of chlorides and sulphates were found to increase in the cultures with high concentrations of Cl or SO_4 which had also retarded plant growth. MCILVANIE (**11**) found that the total nitrogen content of the roots of *Agropyron spicatum* was depleted, in the vegetative stage, to 53% of that present in the formative stage and carbohydrates, 70% of that occurring at the normal minimum; while the greatest relative amounts of reducing sugars were associated with rapid vegetative growth of sucrose with differentiation, and reserve polysaccharides with the brief rest period prior to secondary growth.

Results

GENERAL APPEARANCE

The high-nitrogen plants were very vegetative. The leaves were quite long, broad, green, and soft by comparison with the low-nitrogen plants. The latter were smaller, with narrower, shorter, and stiffer leaves and with some die-back symptoms in the terminal regions of the relatively old or mature leaves. The death of such regions was presumably caused by nitrogen deficiency.

DRY MATTER

Dry matter values reported as mg. per gram of fresh tissue were generally higher in the chlorophyllous sections (nos. 3, 4, and 5) of the leaves

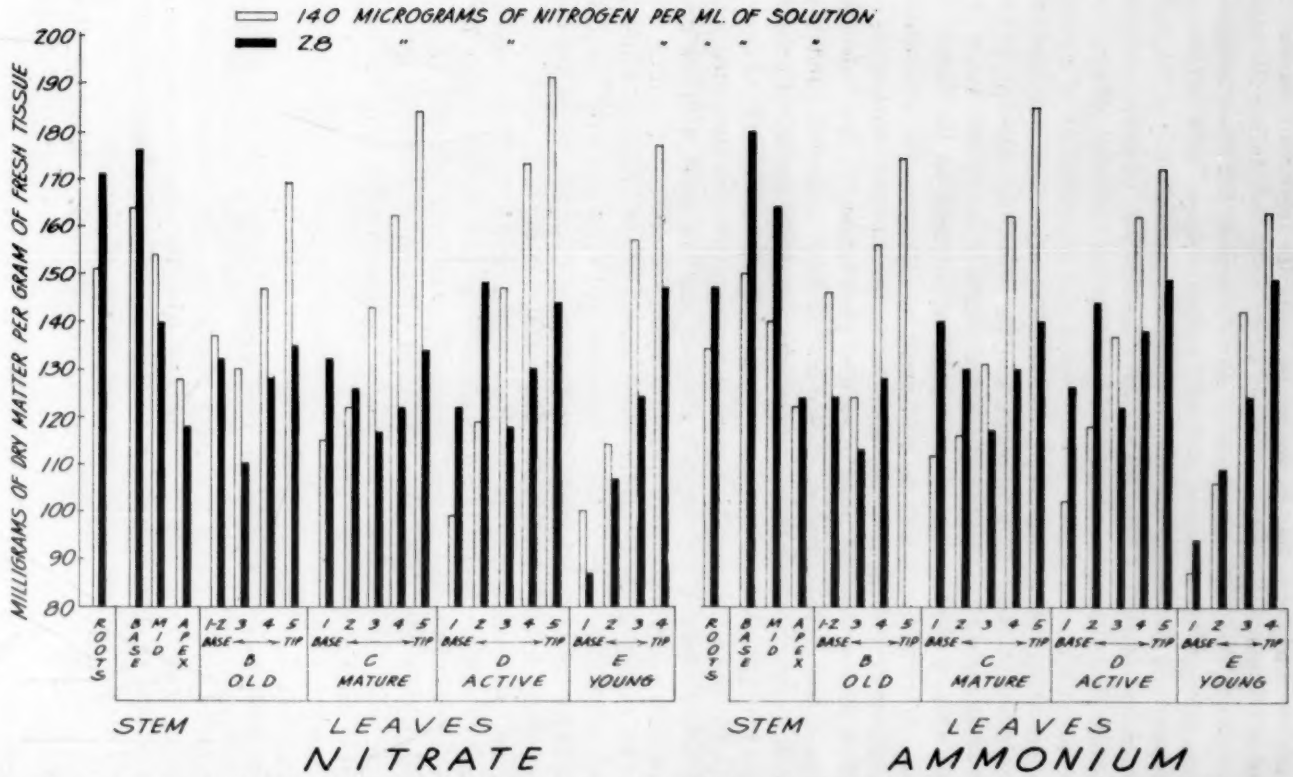

FIG. 1. Dry matter in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

for the high-N than low-N cultures in both nitrate- and ammonium-nitrogen series (fig. 1). Similar values in the combined basal and transitional (nos. 1 and 2) sections of the old (B) leaves which contained some chlorophyll were greater for the high-N than low-N cultures. In the nitrate-N series the basal (no. 1) and transitional (no. 2) sections of the young (E) leaves contained more dry matter for the high-N than low-N cultures. Dry matter values of the stem in the nitrate-N series were greater in the apical and medial but not in the basal sections for the high-N than low-N cultures. Similar values in the ammonium-N series, however, were greater for the low-N than high-N cultures. The roots contained more dry matter for the low-N than high-N cultures in both series.

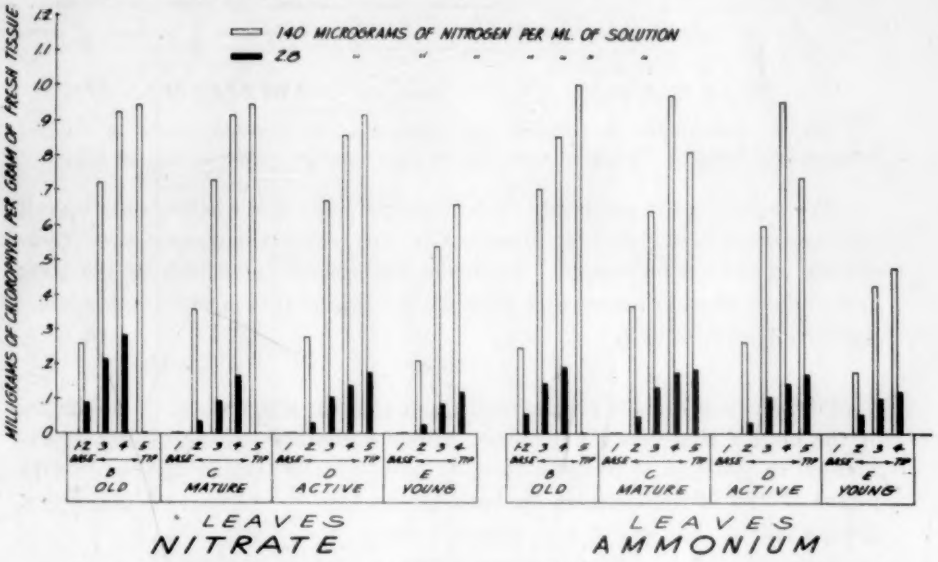

FIG. 2. Chlorophyll in different leaf sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

Additional data indicate that differences in the dry matter content of comparable sections between different cultures resulted mostly from differences in the amounts of organic acids and sugars (table II and figs. 2, 3, 4, and 5).

CHLOROPHYLLOUS PIGMENTS

The chlorophyll and carotenoid content of tissues was greater for the high-N than low-N cultures (figs. 2 and 3). Concentrations of both pigments increased progressively from the basal (no. 1) to the terminal (no. 5) sections of the leaves except in a few sections. In *A. comosus*, as in all other plants, chlorophyll, depending on nitrogen for the synthesis of its molecule and for the proteinaceous stroma and grana of the chloroplasts, increased with greater supplies of nitrogenous fertilizers. The amounts of carotenoids in the various leaf sections correlated with those of chlorophyll, although nitrogen is not a component of the molecules of carotene or xanthophyll.

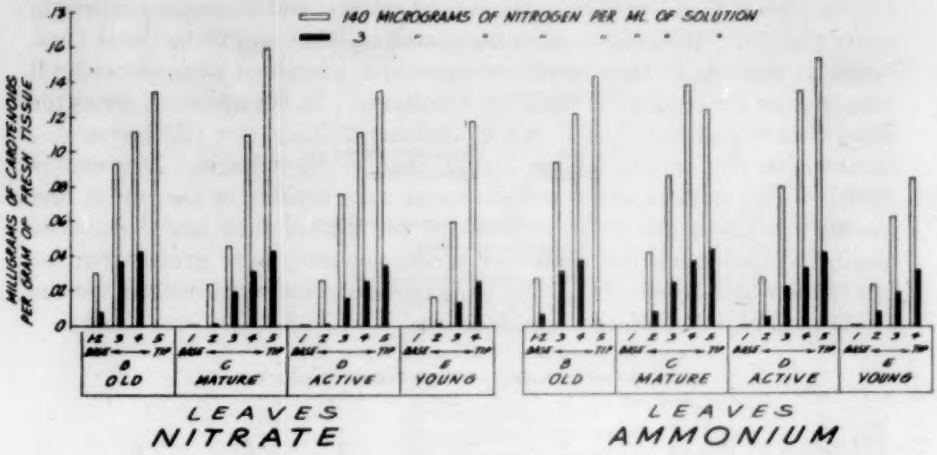

FIG. 3. Carotenoids in different leaf sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

The occurrence in relatively definite proportions in the cell of chlorophyll and carotenoids which are functionally interrelated suggests that their genesis may be synchronous. However, carotenoid formation in the stem and roots of *Daucus carota* and possibly in other plants is presumably independent of chlorophyll.

ACIDITY

Titrable acidity, reported as citric acid (fig. 4), was greater in the leaves of the high-N than low-N cultures. Minor exceptions which cannot be explained were observed in the chlorophyllous sections (no. 3) of the old (B) leaves of the low-N cultures in both series, also in sections of other leaf groups and in the stem of the nitrate-N series.

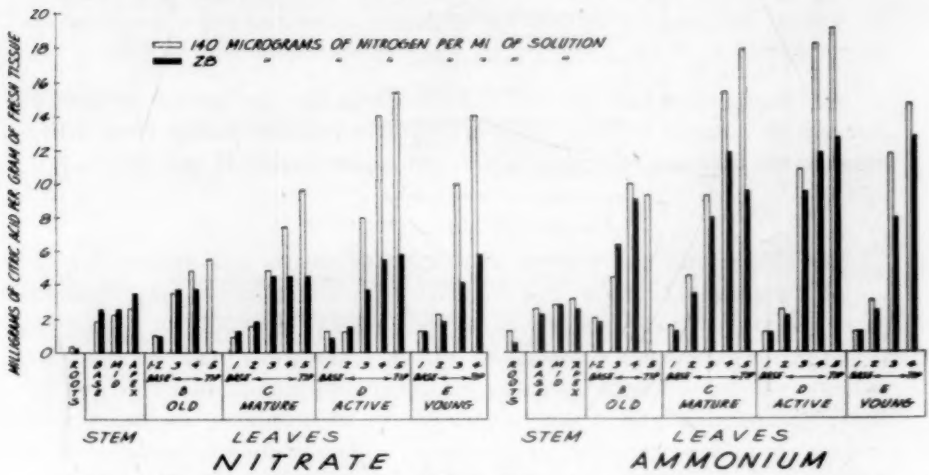

FIG. 4. Titrable acidity, as citric acid, in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

Preliminary studies (**26**) indicated that the organic acid content of chlorophyllous regions in leaves, as determined by weight, increased with greater plant vigor suggesting that the greater organic acid content of the high-N than low-N cultures had probably resulted from increased respiration caused by a higher rate of metabolic activity. Organic acids, presumably products of respiration, accumulated in plant tissues during the night or periods of darkness, possibly through enzymatic oxidation of sugars, and disappeared during the day or periods of light, also through enzymatic reduction to sugars or by further oxidation to carbon dioxide and water.

The acid content of non-chlorophyllous tissues in the basal leaf sections or in the stem or roots is not affected appreciably by diurnal changes, plant

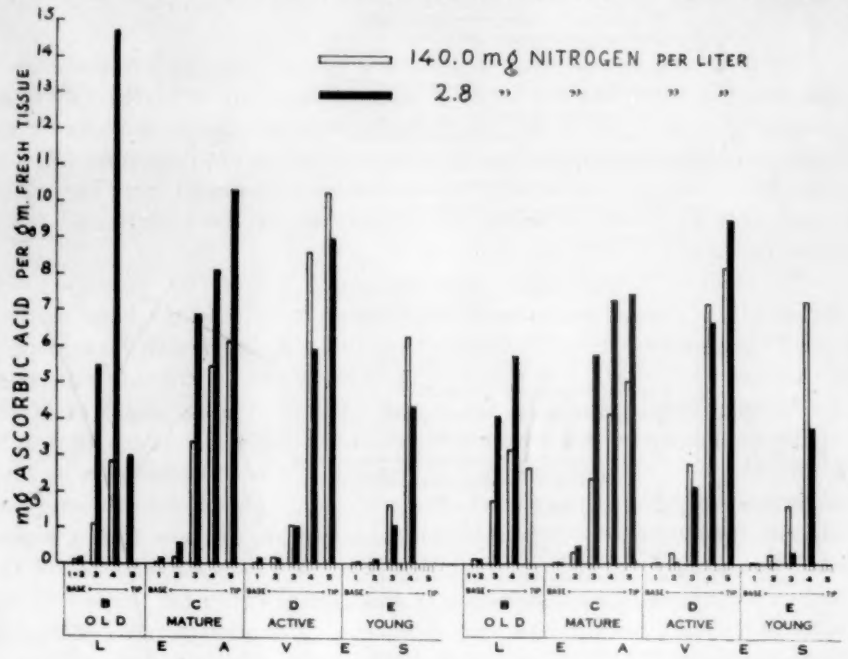

FIG. 5. Ascorbic acid in different leaf sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

vigor, or different kinds of nutrition. Therefore, in view of the accumulation of great amounts of organic acids in the chlorophyllous and exceedingly small amounts in the non-chlorophyllous sections, it would be misleading to associate both sections of the leaves with the same type of biochemical reactions generated during respiration. It is hypothetically possible that respiratory activity in non-chlorophyllous tissues lacking in ascorbic acid, a reducing agent, might involve the complete oxidation of sugars to carbon dioxide and water, whereas in the chlorophyllous tissues, amply supplied with ascorbic acid which is not affected by diurnal changes in the pineapple, sugars may be oxidized mostly to intermediary products, *e.g.*, malic, citric, and other carboxylic acids which tend to accumulate in the tissues.

Ascorbic acid

The amounts of ascorbic acid in the chlorophyllous tissues of the leaves varied considerably between the high-N and low-N cultures (fig. 5). In the young (E) and active (D) leaves ascorbic acid was generally higher for the high-N than low-N cultures, but in the mature (C) and old (B) leaves the low-N contained more than the high-N cultures.

In view of the fact that our knowledge of the physiological functions of ascorbic acid in plant metabolism is limited, no satisfactory explanation can be offered for the greater accumulations of this substance in the young (E) and active (D) leaves of the high-N cultures or the converse in the low-N cultures.

Sugars

Total sugars showed greater accumulations for the high-N than low-N cultures (fig. 6). The gradients of sugar concentration in the tissues increased from the transitional (no. 2) to the terminal (no. 5) sections for the high-N but decreased for the low-N cultures in all except the young (E) leaf groups. In the latter group the sugar gradients increased from the transitional (no. 2) to the terminal (no. 5) section for the high-N and low-N cultures.

The chlorophyllous sections of leaves (nos. 2, 3, 4, and 5), associated with carbohydrate production by photosynthetic activity, and the basal sections (no. 1) composed of meristematic tissues and associated with carbohydrate utilization by formation of new tissues, present two contrasting aspects of the carbohydrate economy of the leaves. Ratios of mean sugar or of the combined sugar plus starch values of the chlorophyllous sections (nos. 2, 3, 4, and 5) to the basal section (no. 1) indicate the relative pressure or level of such carbohydrate substances in the producing (photosynthetic) and consuming (meristematic) regions (table I). The ratios show that a higher level was attained in the chlorophyllous than in the basal sections for the high-N cultures and the opposite or a negative pressure for the low-N cultures in all leaf groups except the young (E). The greater ratios of readily available carbohydrates (sugars and starch) in the chlorophyllous regions of the leaves of the high-N than low-N cultures should be attributed to the higher content of chlorophyll of the former cultures.

Starch

Starch concentrations in fresh plant tissues varied in the different cultures (fig. 7). In general the high-N cultures in the nitrate series contained more starch in the chlorophyllous sections of the leaves and in the stem than the low-N cultures. In the basal (no. 1) and transitional (no. 2) sections of the mature (C), active (D), and young (E) leaves with rapidly growing tissues, however, starch was more abundant for the low-N than high-N cultures. Similar values in the ammonium series were greater for the low-N than the high-N cultures. It was also indicated in former studies (**24**) that

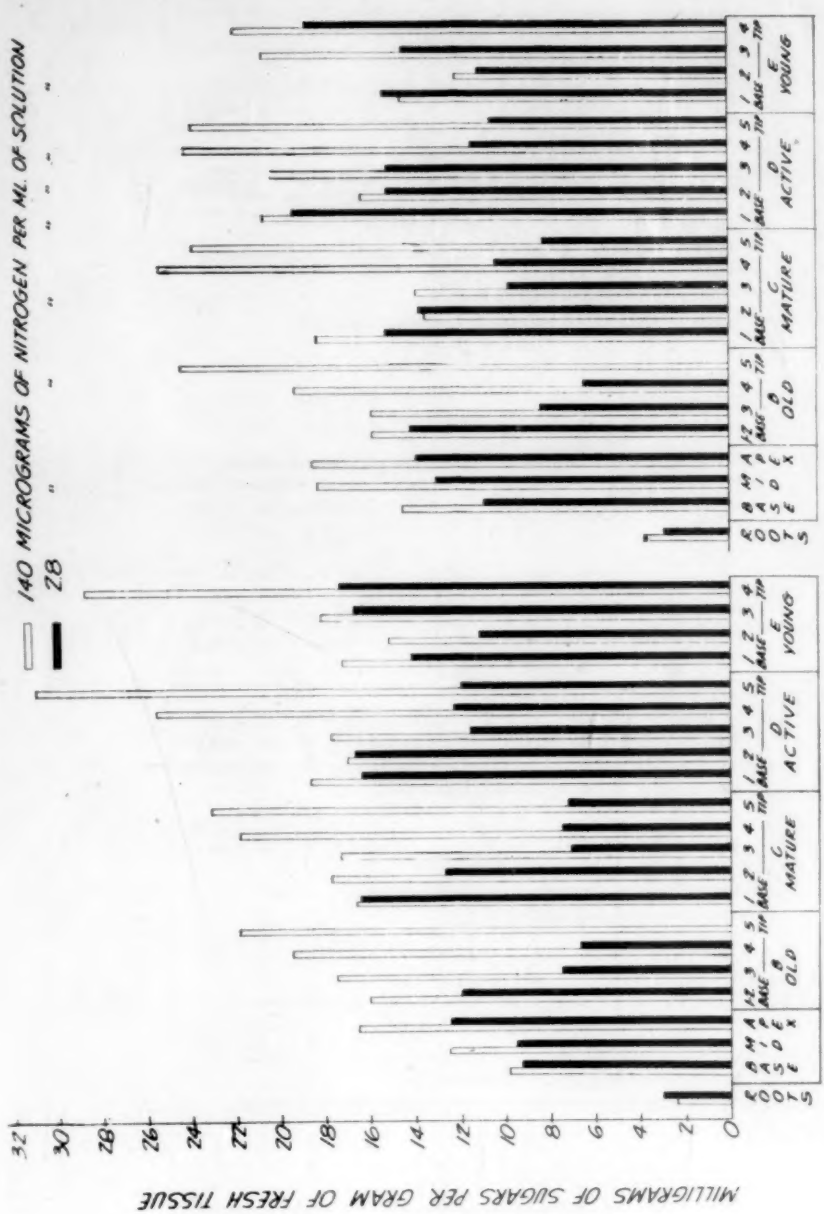

FIG. 6. Sugars in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

TABLE I

MEAN VALUES AND RATIOS OF TOTAL SUGARS OR TOTAL SUGARS PLUS STARCH FOR THE CHLOROPHYLLOUS (NOS. 2, 3, 4, AND 5) AND BASAL (NO. 1) SECTIONS OF DIFFERENT GROUPS OF LEAVES

CARBOHYDRATE FRACTIONS IN LEAVES	NITRATE-N						AMMONIUM-N					
	HIGH-N			LOW-N			HIGH-N			LOW-N		
	CHLOROPHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLOROPHYLLOUS TO BASAL	CHLOROPHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLOROPHYLLOUS TO BASAL	CHLOROPHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLOROPHYLLOUS TO BASAL	CHLOROPHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLOROPHYLLOUS TO BASAL
	mg./gm.	*mg./gm.*		*mg./gm.*	*mg./gm.*		*mg./gm.*	*mg./gm.*		*mg./gm.*	*mg./gm.*	
Total sugars:												
Old (B)	19.50	16.00	1.22	5.00	12.00	0.42	19.50	15.80	1.23	5.60	14.10	0.40
Mature (C)	18.53	16.60	1.12	7.90	16.40	0.48	21.10	18.30	1.15	10.00	15.20	0.66
Active (D)	23.95	18.60	1.29	12.50	16.30	0.77	22.30	20.50	1.09	13.40	19.30	0.69
Young (E)	19.90	17.20	1.16	14.90	14.10	1.06	18.20	14.60	1.25	14.50	15.30	0.95
Starch:												
Old (B)	3.34	7.80	0.43	0.87	1.06	0.82	1.20	0.28	4.28	0.72	1.00	0.72
Mature (C)	3.82	2.26	1.69	2.39	4.86	0.49	1.22	0.15	8.13	2.80	9.72	0.29
Active (D)	4.33	2.20	1.97	6.27	8.23	0.76	0.51	0.28	1.82	3.72	13.70	0.27
Young (E)	4.45	1.48	3.00	1.90	0.41	4.63	0.20	0.28	0.71	1.06	0.62	1.70

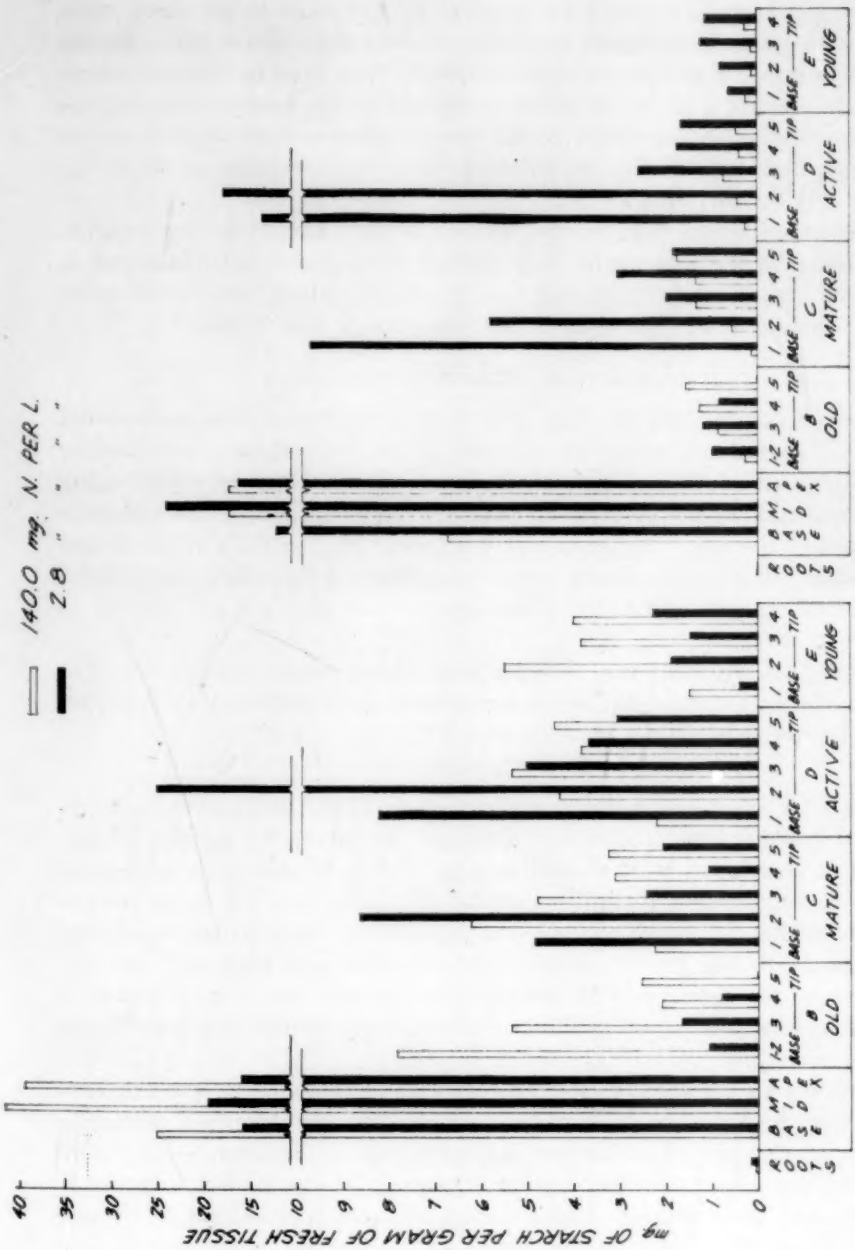

FIG. 7. Starch in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

the lower starch values for the ammonium-N than nitrate-N series might have resulted from a high chloride content in the former series which interfered with optimum metabolic activities. In this study starch ratios for the high-N cultures were greater in the chlorophyllous than in the non-chlorophyllous regions, *e.g.*, the meristematic tissues of the basal sections of the leaves, whereas similar ratios in the low-N cultures were smaller in the chlorophyllous than in the non-chlorophyllous basal sections except in the young (E) leaves (table I).

The data indicate that reduced growth activity caused by low supplies of nitrogen in the substratum may produce great starch accumulations in the basal (no. 1) and transitional (no. 2) sections which result from a low rate of carbohydrate utilization in the formation of new tissues.

Hemicellulose, cellulose, and lignin

Former studies (**23, 25**) indicated that no significant changes resulted from differential treatments of nutrition in the hemicellulose, cellulose, or lignin content of tissues, possibly because these substances representing structural and not energy-yielding units are relatively inert to the metabolic activities of the cell. Winkler and Williams (**30**), in *Vitis vinifera,* and Wadleigh (**29**), in *Gossypium barbadense,* observed that the hemicelluloses are not affected by nutritional conditions because they are not utilized as sources of energy.

Total hemicellulose, and cellulose plus lignin values estimated on the basis of former findings (**24**) are compared with other carbohydrate fractions of the tissues (table II).

Discussion

The relation of nitrogen to carbohydrate synthesis and accumulation in plant tissues has been studied and discussed extensively by various investigators in association with vegetative growth and fructification under the title "carbon/nitrogen ratios." Thomas (**27**), reviewing the entire subject of carbon/nitrogen ratios, concluded as follows: (a) Carbohydrates increase in tissues with low growth activity and decrease with high activity; (b) nitrogen of the tissues is high before active growth, but it decreases with active growth, and (c) no specific carbon/nitrogen ratios have been found for any response.

Data herein presented cannot be evaluated in the light of Thomas' conclusions with respect to fruit yields because they are concerned primarily with vegetative growth and not with fructification. However, certain comparisons concerned with the vegetative growth of the high-N and low-N cultures and their content of total sugars and starch reveal the following: Sugar levels were lower in the basal (no. 1) sections with meristematic tissues than in the chlorophyllous sections of the leaves (nos. 2, 3, 4, and 5) for the high-N cultures with a high rate of vegetative growth and great carbohydrate utilization (fig. 8). Similar levels for the low-N cultures with reduced vegetative growth were reversed, being higher in the basal (no. 1)

TABLE II

TOTAL AMOUNTS OF OBSERVED DRY MATTER AND RECOVERED SUBSTANCES PER PLANT (IN GRAMS AND IN PERCENTAGE OF OBSERVED DRY MATTER) IN ONE-YEAR-OLD *Ananas comosus* GROWN IN SOLUTION CULTURES WITH 140.0 OR 2.8 MILLIGRAMS OF NITROGEN PER LITER EITHER AS NITRATE OR AMMONIUM

DRY MATTER	NITRATE-N								AMMONIUM-N							
	HIGH-N				LOW-N				HIGH-N				LOW-N			
	PLANT	LEAVES	STEM	ROOTS	PLANT	LEAVES	STEM	ROOTS	PLANT	LEAVES	STEM	ROOTS	PLANT	LEAVES	STEM	ROOTS
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Cellulose + lignin*	139.00	118.00	7.85	21.00	87.00	55.40	4.04	34.30	106.63	92.20	5.43	9.00	107.50	76.80	5.26	28.25
Hemicellulose*	72.00	58.40	6.53	10.75	45.20	29.50	2.94	17.60	59.61	49.50	4.51	5.00	44.40	32.80	3.39	15.20
Sugar	65.45	60.11	4.57	0.77	23.26	20.31	1.53	1.42	58.27	53.30	4.34	0.63	28.13	24.84	2.12	1.17
Starch	25.50	11.52	13.97	0.01	8.53	5.95	2.57	0.01	5.28	1.74	3.53	0.01	8.74	5.66	3.05	0.03
Citric acid	23.62	22.64	0.88	0.10	7.02	6.50	0.42	0.10	30.30	29.36	0.74	0.20	16.63	15.95	0.44	0.24
Ascorbic acid	11.94	11.94	.....	.....	7.26	7.26	.....	.....	9.24	9.24	.....	.....	7.82	7.82	.....	.....
Chlorophyll, P.	2.21	2.21	.....	.....	0.22	0.22	.....	.....	1.76	1.76	.....	.....	0.27	0.27	.....	.....
Org. N (×6.25)	54.10	45.53	5.24	3.33	11.20	7.88	1.00	2.32	61.00	55.80	3.67	1.53	11.62	8.72	1.08	1.82
Ash	44.34	37.12	5.03	2.19	31.65	26.87	2.19	2.59	29.23	25.73	2.65	0.85	35.50	31.26	2.40	1.84
Total recovered	438.16	367.47	44.07	38.15	221.34	159.89	14.69	58.34	361.32	318.63	24.87	17.22	265.61	204.12	17.74	48.55
Total observed	551.54	448.09	54.85	48.60	321.00	218.00	21.94	81.00	436.63	381.03	34.70	20.90	345.74	260.32	26.21	59.20
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Cellulose + lignin*	25.52	26.40	14.28	43.25	27.08	25.40	18.40	42.30	24.42	24.20	15.65	43.00	31.10	29.08	21.05	47.65
Hemicellulose*	13.04	13.00	11.90	22.15	14.68	13.55	13.40	21.70	13.65	13.00	13.00	23.90	14.30	12.60	12.95	25.50
Sugar	11.90	13.42	8.34	1.60	7.25	9.30	7.00	1.75	12.73	13.20	12.50	3.00	8.14	9.52	8.10	1.98
Starch	4.64	2.57	25.50	0.02	2.66	2.73	11.70	0.01	1.21	0.46	10.20	0.05	2.53	2.18	11.65	0.05
Citric acid	4.27	5.04	1.60	0.20	2.18	2.98	1.92	0.12	6.95	7.70	2.13	0.96	4.81	6.15	1.68	0.40
Ascorbic acid	2.17	2.64	.....	.....	2.26	3.33	.....	.....	2.12	2.42	.....	.....	2.26	3.01	.....	.....
Chlorophyll, P.	0.40	0.49	.....	.....	0.07	0.10	.....	.....	0.40	0.46	.....	.....	0.08	0.10	.....	.....
Org. N (×6.25)	9.80	10.16	9.55	6.85	3.50	3.62	4.60	2.87	14.00	14.65	10.60	7.30	3.36	3.36	4.12	3.08
Ash	8.50	8.29	9.17	4.50	9.82	12.33	10.00	3.20	6.70	6.77	7.64	4.10	10.30	12.00	9.16	3.11
Total recovered	80.24	82.01	80.34	78.57	68.90	73.34	67.02	71.95	82.18	82.86	71.72	82.31	76.88	78.00	68.77	81.77

* Estimated values on the basis of previous analyses (**24**).

than in the chlorophyllous (nos. 2, 3, 4, and 5) sections of the leaves, indicating a low rate of carbohydrate utilization and of synthesis. Differences in sugar levels between the basal (no. 1) and chlorophyllous (nos. 2, 3, 4, and 5) sections of the mature (C) and active (D) leaves were very small for the high-N cultures in the ammonium series, possibly because of interference with sugar utilization for the formation of new tissues by high chloride concentrations. Starch accumulated in greater amounts in the transitional (no. 2) and basal (no. 1) leaf sections of the low-N than high-N cultures, further corroborating the association of higher amounts of starch with plants of low vegetative growth. The relationship of higher amounts

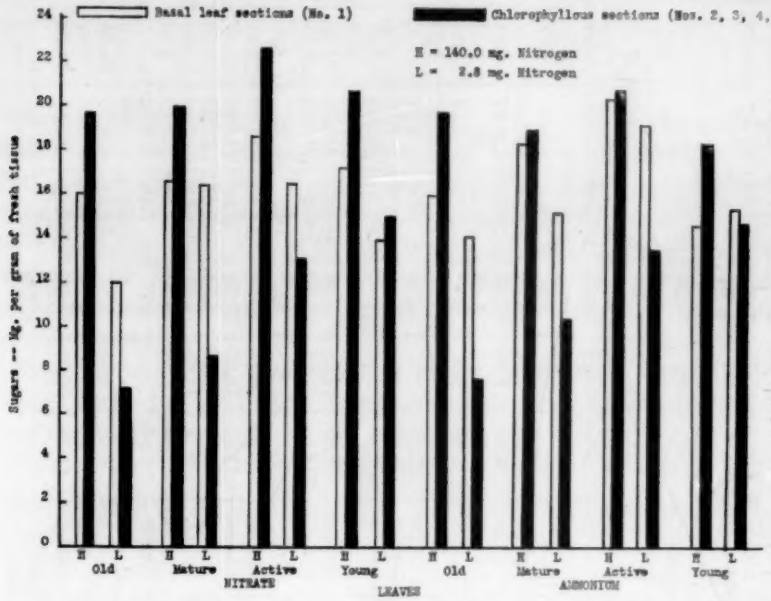

FIG. 8. Sugars, as mg. per gm. of fresh tissue, in the basal (meristematic) and chlorophyllous (photosynthetic) sections of the leaves of high-N and low-N cultures.

of available carbohydrates (sugar and starch) with plants of low vegetative growth are in harmony with THOMAS' postulation *a*. (Postulation *b* will be discussed in a subsequent publication.)

Sugar and starch were higher in the basal (no. 1) and transitional (no. 2) than in the chlorophyllous (nos. 3, 4, and 5) leaf sections of the low-N cultures, whereas in the high-N cultures the levels were lower (figs. 6 and 7). These should be attributed to the restricted nitrogen supplies of the former cultures which caused reduction in the rate of growth and resulted in carbohydrate accumulations. Also, the higher chlorophyll content of the high-N cultures contributed toward greater carbohydrate synthesis. Results thus far indicate that physiological conditions which prompt reduction of vegetative growth also favor carbohydrate accumulations in plant tissues. Retardation of vegetative growth may result from insufficient amounts of all

essential mineral nutrients, water, or from traumatic and pathological factors; the latter might cause carbohydrate accumulations by interfering with the speed of carbohydrate translocation to other plant tissues or utilization for the formation of new tissues. Carbon dioxide, light, and temperature in insufficient amounts, although retarding vegetative growth, may cause carbohydrate depletion because they are directly associated with photosynthesis.

The relations of organic nitrogen fractions to carbohydrates have been studied by WOOD and PETRIE (**31**) who concluded: "there is no evidence that the steady state relations between proteins and amino acids may differ according to the carbohydrate concentrations."

Differences in various carbohydrates or other tissue contents between high-N and low-N cultures were greater for the former cultures when calculated on the basis of total plant weight than per gram of tissue because plant weights were greater for the former than latter cultures. For example, total sugars per plant were 181.5% and 107.0% greater in the high-N than low-N cultures for the nitrate-N and ammonium-N series, respectively (table II). Similar values per gram of fresh tissue (mean of all values) were 58.0% in favor of the high-N cultures for the nitrate-N and 45.8% for the ammonium-N series (fig. 6). However, the greater sugar concentrations (per gram of tissue) in the high-N than low-N cultures suggest that they resulted from a higher chlorophyll content in the former than latter cultures, but not from a reduced rate of vegetative growth.

Starch accumulations differed from sugars in relation to the chlorophyll content of the plants and rate of vegetative growth (table II). Between high-N and low-N cultures in the nitrate-N series starch differences per plant of fresh weight were 200.0% in favor of the former cultures, and in the ammonium-N series 65.5% in favor of the latter cultures; the lower starch content of the high-N cultures in this series resulted presumably from chloride toxicity. Similar differences per gram of fresh tissue (mean of all values) were 34.0% in favor of the high-N cultures in the nitrate-N series and 132.5% in favor of the low-N cultures in the ammonium-N series (fig. 7). Starch accumulations in the nitrate-N series were directly related to the chlorophyll content of the cultures, but in the ammonium-N series they were apparently influenced more by reduction in vegetative growth than by differences in the chlorophyll content of the tissues.

The data suggest that the small and insufficient nitrogen content of the low-N cultures decreased indirectly the sugar levels of the chlorophyllous sections by limiting chlorophyll synthesis within the available nitrogen supplies.

Preliminary studies (**26**) showed that titrable acidity in the chlorophyllous sections of leaves increases during the night or in darkness and decreases during the day or in light, and that such acidity associated presumably with metabolic activity correlated with plant vigor as measured by weight. Between high-N and low-N cultures acidity differences in the leaves

(in favor of the high-N cultures) were 85.3% for the nitrate-N and 29.3% for the ammonium-N series (fig. 4).

Ascorbic acid differences per organ (leaves) between high-N and low-N cultures were 64.5% and 18.2% in favor of the former cultures for the nitrate-N and ammonium-N cultures, respectively (table II). However, similar differences per gram of tissue (but in favor of the low-N cultures) were 43.0% for the nitrate-N and 20.6% for the ammonium-N cultures (fig. 5). Although total ascorbic acid per plant weight was greater in the high-N than low-N cultures, actual concentrations were lower in the former than latter cultures (table II and fig. 5). Such differences cannot be explained satisfactorily because of our limited knowledge of the physiological functions of ascorbic acid in plant metabolism.

Available information (**5, 6, 7, 23, 24**) indicates a significant parallelism between concentrations of ascorbic acid and of chlorophyll in different plants. However, MIRIMANOFF'S (**12, 13**) and NEISH'S (**16, 17**) studies present a different picture of the ascorbic acid and chlorophyll relationships; the former claiming a more probable association of ascorbic acid with flavo-

TABLE III

CHLOROPHYLL AND ASCORBIC ACID CONTENT AS MG. PER GM. OF FRESH TISSUE IN PLUS- AND MINUS-CHLOROPHYLL LEAF SECTIONS AND RATIO OF THE SAME OF (VARIEGATED) *A. bracteatus*

LEAF SECTIONS	CHLOROPHYLL	ASCORBIC ACID	ASCORBIC ACID/CHLOROPHYLL
	mg./gm.	*mg./gm.*	
Plus chlorophyll	0.501	1.20	2.40
Minus chlorophyll	0.064	0.54	8.43

nols than with chlorophyll, and the latter asserting that determination of ascorbic acid in separate chloroplasts did not show much difference between that and the leaves as a whole.

Determinations of ascorbic acid in adjoining leaf sections of variegated leaves of *Ananas bracteatus* show relations of ascorbic acid to chlorophyll for the plus- and minus-chlorophyll sections (table III).

Ratios of ascorbic acid to chlorophyll were greater for the minus- than for the plus-chlorophyll sections, suggesting that some other factor in addition to chlorophyll might have contributed toward ascorbic acid synthesis, or that its utilization under chlorotic conditions might not have been so great as under conditions of greater chlorophyll content in the tissues. Also, it is possible that ascorbic acid, being very soluble in the sap, may move by diffusion from the plus- to the minus-chlorophyll regions of the leaves, enriching the latter. Although there is some relationship between ascorbic acid and chlorophyll content of tissues, the amounts are not strictly proportional (**23, 26**).

GUHA and GHOSH (**8**) associate mannose as the raw material for ascorbic acid synthesis while BUKATSCH (**2**) and REID (**20**) claimed that sugars produced photosynthetically in leaves are responsible for increasing ascorbic acid content of the tissues of these organs.

Mean ascorbic acid values in the nitrate-N series were 3.63 mg. for the high-N and 5.26 mg. per gram for the low-N cultures, and in the ammonium-N series, they were 3.35 mg. for the high-N and 4.10 mg. for the low-N cultures. Mean chlorophyll values in the nitrate-N series were 0.622 mg. and 0.123 mg. per gram for the high-N and low-N cultures, respectively, while in the ammonium-N series they were 0.614 mg. for the high-N and 0.118 mg. for the low-N cultures. Ratios of ascorbic acid to chlorophyll in the nitrate-N series were 5.84 and 42.80 for the high-N and low-N cultures, respectively, and in the ammonium-N series, 4.47 for the high-N and 34.75 for the low-N cultures. Hence, the ratios of ascorbic acid to chlorophyll, indicating greater accumulations for the low-N than high-N cultures, may suggest some association of nitrogen with ascorbic acid in the metabolism of various nitrogenous compounds. MITCHELL (**14**) reported that v. Euler, Karrer, and Zehender found ascorbic acid or dehydroascorbic acid capable of dehydrating leucine with the formation of ammonia and strongly reducing volatile substances, and Abderhalden observed that in the presence of iron and oxygen, ascorbic acid deaminized and decarboxylated many amino acids.

Summary

The carbohydrate economy of *A. comosus* was studied in relation to high and low levels of nitrogen (140.0 and 2.8 mg. per liter) supplied to nutrient solutions either as nitrate or ammonium ions with the following results:

1. The percentage of dry matter in the chlorophyllous regions of healthy leaves was greater for the high-N cultures than for the low-N cultures, but in the non-chlorophyllous (basal) and transitional regions, the reverse was true. Similar values in the stem were greater for the high-N cultures in the nitrate series except in the basal section, but lower for the same cultures in the ammonium series.

2. Chlorophyll concentrations in leaf tissues were considerably greater for the high-N than low-N cultures. Carotenoids were also greater for the high-N than low-N cultures.

3. Titrable acidity, reported as citric acid, which by respiratory activity in the chlorophyllous leaf tissues increased during the night and decreased during the day, was greater in the high-N than low-N cultures and correlated in general with plant weights.

4. Ascorbic acid, limited mostly to the chlorophyllous regions of the leaves, varied in amounts between young or active and old or mature leaves. The amounts of ascorbic acid in the young or active leaves were greater for high-N cultures; but in the old or mature leaves they were greater for the low-N cultures.

5. Sugars in the leaves and stem were generally greater for the high-N than for the low-N cultures. The gradients of sugar concentrations increased from the transitional to the terminal leaf sections for the high-N cultures, but decreased for the low-N cultures except in the very young leaves.

6. Starch of the chlorophyllous sections of the leaves and of the stem, in the nitrate series, was greater for the high-N than low-N cultures, but in the non-chlorophyllous and transitional sections of the leaves, it was greater for the low-N cultures. Nearly all leaf and stem sections in the ammonium series contained more starch for the low-N than high-N cultures.

7. The relations of nitrogen to vegetative growth, sugar, starch, and organic acid levels in the tissues of plants were discussed in some detail.

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THE VITAMIN CONTENT OF SOYBEANS AND SOYBEAN SPROUTS AS A FUNCTION OF GERMINATION TIME

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(WITH THREE FIGURES)

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The investigation upon which this report is based was made for the purpose of comparing the vitamin content of mature unsprouted soybeans of the Bansei variety with that of the same variety of soybeans after different periods of sprouting under controlled conditions, by a method suitable for yielding an edible product. Since the usual practice in oriental countries is to use the hypocotyls and to discard the cotyledons, the relative distribution of vitamins was ascertained separately in these portions of the sprouted soybeans after 54 hours of germination—the optimum period from the point of view of quantity coupled with organoleptic properties. The study included analyses of carotene, thiamin, riboflavin, niacin, and ascorbic acid content.

Sprouting was carried out under controlled conditions, following a technique found during preliminary trials to be favorable for edibility.

Historical review

Numerous studies have been made during the past three decades on one or more aspects of the nutritive value of sprouted soybeans and other legumes or grains; few studies, however, have dealt with products which have been prepared under conditions conducive to a high degree of edibility, and these have not been concerned, except in a few cases, with the relative merits of the hypocotyls and the cotyledons.

Early investigations in this field were confined chiefly to ascorbic acid content, using experimental animals for assessment purposes. Thus, in 1915, CHICK and DELF (**12**) demonstrated the antiscorbutic potency of germinated peas and lentils, and in 1917 WILTSHIRE (**29**) showed the same property in Haricot beans. EMBREY (**15**) in 1921 and SANTOS (**26**) in 1922 reported that the vitamin B content of Mung beans increased during germination.

From 1928 to 1940, MILLER and HAIR (**23**) studied the content of several vitamins in Mung beans, BOGARD and HUGHES (**6**) the ascorbic acid content of oats at different stages of sprouting, LEE and READ (**19**) the ascorbic acid content of sprouted soybeans in light and in darkness using the analytical method of BESSEY and KING (**4**), LEE (**18**) the ascorbic acid content of sprouted peas including the distribution of this vitamin through the cotyledons and the hypocotyls, ROSE and PHIPARD (**25**) the B vitamins in sprouted

peas and YEH (30) the thiamin content of unsprouted and sprouted Mung beans.

In 1942, BHAGVAT and NARASINGA RAO (5) showed an increase in ascorbic acid in sprouted Mung beans, reaching a maximum at 30 to 48 hours. Separate analyses were performed on the hypocotyls and cotyledons; the latter were found to be richer at the early stages of development.

In 1943, LUGG and WELLER (20), and BEESKOW (2) studied the ascorbic acid content of sprouted Mung beans and LANE (11) reported on the riboflavin content of black-eyed peas. Increases were reported after certain lapses of sprouting time in comparison with the fresh dry bean.

In papers reported from 1942 to 1945, Burkholder and associates described vitamin changes during the sprouting of beans and grains. Thus BURKHOLDER and MCVEIGH (9) published values for thiamin, riboflavin, niacin, and biotin on dry and germinated Mung beans, lima beans, soybeans, peas, and wheat, although techniques of sprouting compatible with edibility were not followed. For soybeans germinated in liquid culture, there was usually a loss in vitamins during a preliminary soaking in a solution containing a low concentration of sodium hypochlorite. After four days of sprouting in a liquid culture the riboflavin had doubled and the niacin had increased one and one-half times in comparison with the initial dry bean; thiamin showed little increase.

BURKHOLDER (7) reported that grains—barley, corn, oats, and wheat—apparently synthesized certain of the vitamins during sprouting.

BURKHOLDER (8) gave the vitamin content of different varieties of soybeans. On the basis of micrograms per gram of dry matter, the Bansei variety contained 8.4 μg. of thiamin, 2.4 μg. of riboflavin, and 23 μg. of niacin per gram. The ascorbic acid value was found to be 0.21 milligram per gram of green raw beans. MCVEIGH (21), in the same laboratory with BURKHOLDER, made studies on various parts of the oat seedling grown in the dark for five days, and reported marked increases in riboflavin and niacin, but none in thiamin, measured in content per seedling.

BURKHOLDER and MCVEIGH (10) studied Canner King peas, the Mung bean commonly used in chop suey, and seven varieties of field soybeans. On a dry weight basis, riboflavin, niacin, and ascorbic acid increased greatly during germination, although thiamin showed little change.

FRENCH *et al.* (16) reported in 1944 on changes during sprouting in the vitamin content of peas, lima and kidney beans, as well as six varieties of soybeans. Some signs of germination were observed at the end of 24 hours, and, in most cases, sprouts were fully evident in 48 hours. The rates of germination and of vitamin formation were observed to be directly correlated with the temperature of the room.

Germination caused a marked increase in ascorbic acid, a fair increase in riboflavin and niacin, and none in thiamin. Average values for six varieties of soybeans after 72 hours of sprouting were 13.5, 0.29, 0.46, and 1.09 milligrams per 100 grams of the moist legume, for ascorbic acid, thiamin, riboflavin, and niacin, respectively.

Methods

One lot of Bansei soybeans was used throughout the study. In preparation for sprouting, the soybeans were washed four times with distilled water at 20° to 21° C., and then were hand-sorted. Broken fragments as well as beans with broken seed coats were discarded. The selected beans were soaked for 10 hours in a solution consisting of dilute calcium hypochlorite (5.5 grams per 10 liters of distilled water) to prevent mold growth. At the close of the soaking period, the soybeans were drained, washed with water at about 21° C., and placed in sprouting vessels which were kept in darkness in a constant temperature cabinet at 28° C. throughout the germination period. The conditions for sprouting were those found by preliminary trials to give a good edible product. Temperatures higher than those of the room were suggested by the work of EDWARDS (**14**), although his recommendation of 92° to 100°F. was not found to give so good a product as the temperatures used.

Enamel pans about 30 centimeters in diameter with handles on opposite sides served as sprouting vessels. The soybeans were placed on a double layer of cheesecloth suspended between the two handles. This provided the necessary drainage and permitted a layer of about three inches of water to be maintained below the cheesecloth to furnish water vapor. A piece of cheesecloth moistened with distilled water was placed over the beans, which were watered three times daily. Non-viable beans were removed as the sprouting progressed.

Two lots of soybeans were germinated, and analyses on duplicate samples were made in each case on the initial dry soybean, on the soybean after the 10-hour soaking period, and after 24, 48, 54, and 72 hours of germination under the conditions mentioned.

The method of WALL and KELLEY (**28**) as modified by COTTON (**13**) was used in the analysis of carotene. The modification includes immediate immersion and heating of food samples, the provision for the control of alcohol concentration in the lower phase during phasic separation according to AUSTIN and SHIPTON (**1**), and the recycling of the petroleum ether in the chromatographic separation. These changes permit better comparisons between the concentrations of carotene in raw and in processed fruits and vegetables.

Thiamin was determined by the Phycomyces method of HAMNER, STEWART, and MATRONE (**17**), riboflavin by the microbiological assay method of SNELL and STRONG (**27**), niacin by the procedure of MELNICK, OSER, and SIEGEL (**22**), and ascorbic acid (reduced and dehydro) by a combination of the methods of BESSEY (**3**) and of MORELL (**24**).

In making individual analyses of the dicotyledons and hypocotyls, the latter were separated carefully by hand. Moisture content was determined by drying in a 65° C. constant temperature oven for three days.

Results

The relative sizes of the initial dry and soaked soybeans, together with the comparative lengths of the sprouts after the different germination times are given (fig. 1). Figure 2 gives the number of soybeans in a 25-gram sample, initially, after soaking, and after the various germination periods;

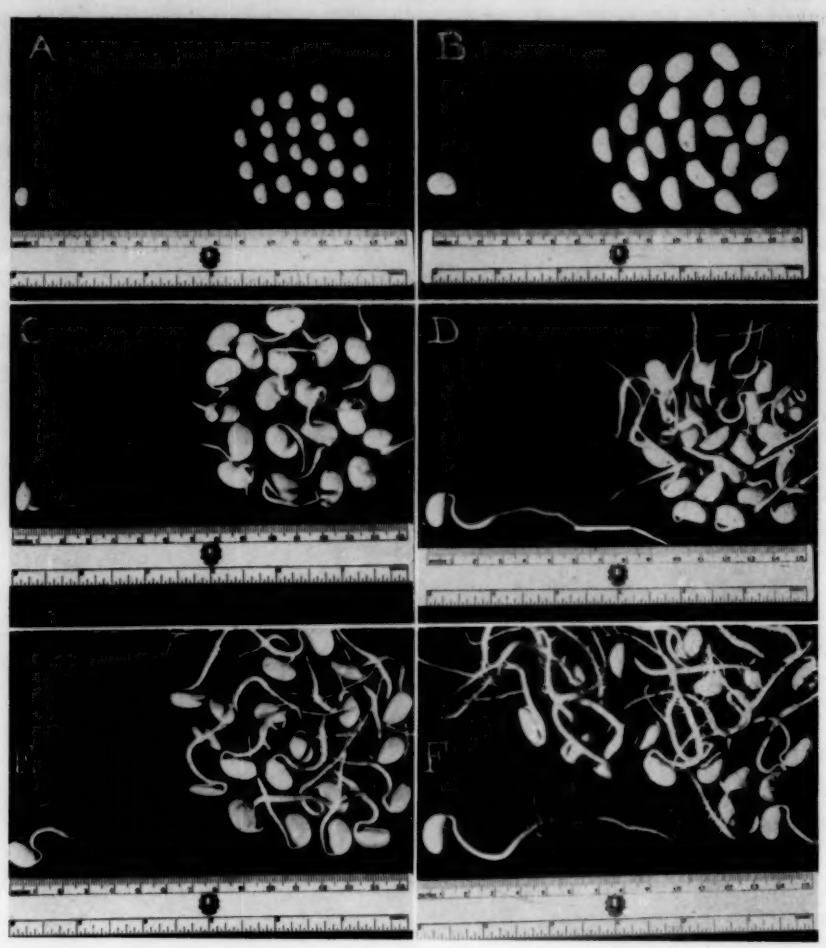

FIG. 1, A–F. Relative sizes of initial dry soybeans (A), of soybeans after soaking (B), and comparative lengths of sprouts after periods of germination of 24 hours (C), 48 hours (D), 54 hours (E), and 72 hours (F).

it gives, also, the vitamin content per soybean in the soaked bean, and in the sprouted bean after the four specified germination periods. Table I gives the vitamin content, calculated on the dry weight basis, of two sets of Bansei soybeans, after soaking and after four germination periods.

On an individual bean basis, there were but slight changes during the 10-hour soaking period in the content of those vitamins for which tests were

made, except for slight losses in thiamin and in reduced ascorbic acid, and slight gains in the dehydro form of ascorbic acid. The beans swelled considerably, however, and the number of soybeans per 25-gram sample decreased from 136 in the initial dry state to 56 of the soaked product in the first series, and from 137.5 to 55.5 in the second. The number of beans per 25-gram sample was reduced gradually, but only slightly during germination.

On the basis of average concentration per soybean, in the first series, carotene had almost doubled in 48 germination hours, and it had increased

TABLE I

VITAMIN CONTENT, DRY BASIS, OF TWO SETS OF BANSEI SOYBEANS AFTER SOAKING AND AFTER FOUR GERMINATION PERIODS

HOURS OF GERMINATION	CAROTENE	THIAMIN	RIBOFLAVIN	NIACIN	ASCORBIC ACID	
					REDUCED	TOTAL
	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.
First series						
0*	1.2	12.8	2.1	27.6	8.7	88.3
24	1.2	17.2	2.8	34.1	198.0	273.6
48	2.2	9.0	3.0	50.8	429.0	517.0
54	3.8	8.4	4.5	45.8	469.4	627.5
72	4.3	11.0	5.6	45.8	354.9	626.3
Second series						
0*	1.3	14.0	2.1	21.0	0.0	108.1
24	0.8	19.7	1.8	21.6	141.6	199.8
48	1.9	9.7	2.7	33.6	421.6	582.3
54	1.6	9.5	4.0	30.0	488.6	599.7
72	2.0	13.4	5.0	50.5	557.9	705.2

* Soybeans after 10 hours of soaking in distilled water containing 5.5 grams of calcium hypochlorite in 10 liters of solution preparatory to soaking.

2.8-fold in 54 and 3.4-fold in 72 hours. In the second series, carotene increases were much lower; the maximum concentration was reached at 48 hours, with a 1.4-fold increase in 48 hours and no further change through 72 hours. The same trends were shown when the carotene concentrations were calculated on a dry weight basis.

Thiamin showed a slight gain in concentration during the first 24 hours of germination, followed by a loss through 54 hours, and a gain which brought the concentration close to the original between 54 and 72 hours. This trend was shown in both series, whether the calculation was made on an average per soybean or a dry matter basis. Further tests for this vitamin in other germination series showed that a periodic loss was followed by a gain in thiamin throughout the germination period of the soybean investigated.

Riboflavin showed a 1.9-fold increase in 54 hours of sprouting in the first series, calculated on an average per soybean basis, and a 2.5-fold increase in 72 hours (fig. 2). In the second series the increases were 1.8- and

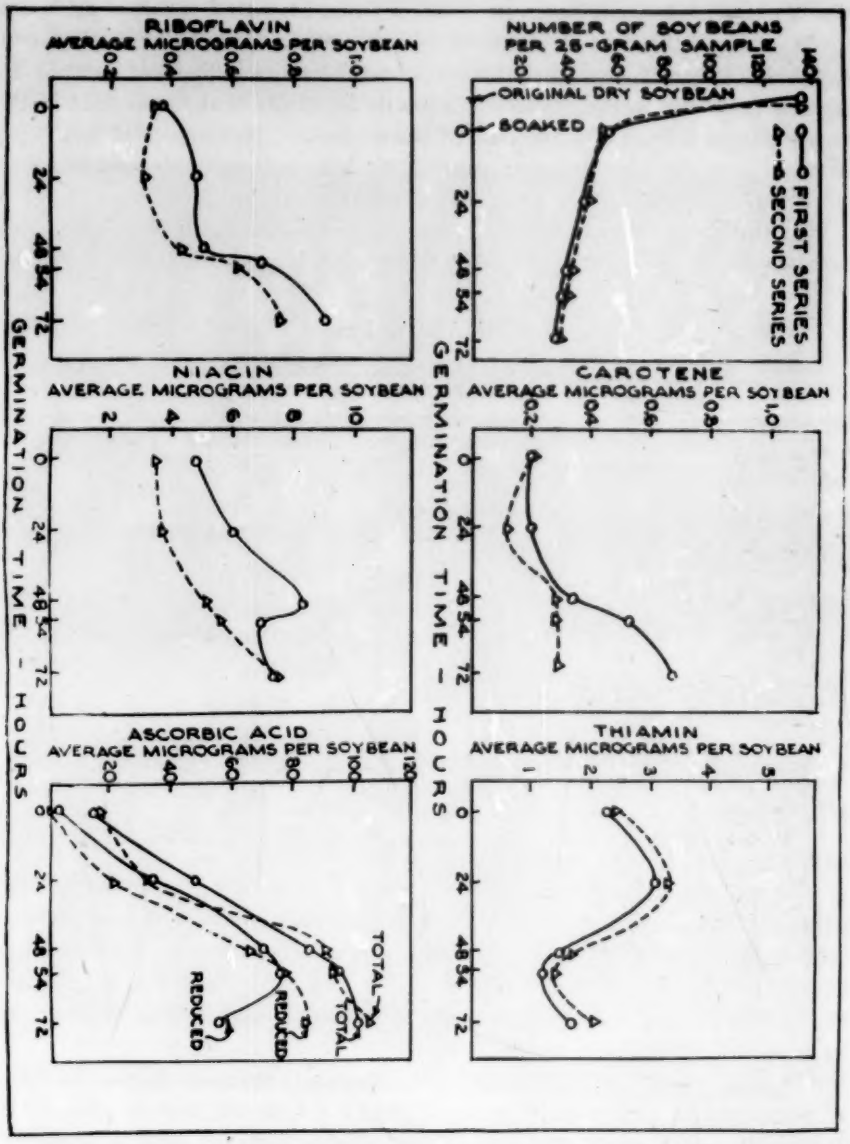

FIG. 2. Comparative number of soybeans in a 25-gram sample initially, after soaking, and after the respective germination times, average content per soybean (micrograms) of carotene, thiamin, riboflavin, niacin, and ascorbic acid (reduced and total) of the soaked bean, and of the bean after 24, 48, 54, and 72 hours of germination in the dark under conditions suitable for producing an edible product (28° C. in a cabinet providing temperature control).

2.1-fold for these respective germination periods. On a dry weight basis, the increases were 2.0- and 2.6-fold, and 1.9- and 2.4-fold for the two series and the 54-hour and 72-hour times of germination, respectively.

In both series of tests, the average niacin content per soybean was approximately doubled in 72 hours of sprouting. In the first series the rate of increase was greater for the intermediate times, with a minimum reached at 48 hours. In the second the increase was steady through the 72 hours of observations. Calculations on a dry weight basis showed the same general trends.

Ascorbic acid content showed the most marked increases during germination of any of the vitamins studied. In one series the soaked product contained an average of 8.7 micrograms of reduced and 88.3 of total (sum of reduced and dehydro) ascorbic acid per soybean; in the second series, there was no measurable amount of the reduced and 108.1 micrograms of

TABLE II

RELATIVE DISTRIBUTION OF DIFFERENT VITAMINS IN THE HYPOCOTYLS AND COTYLEDONS OF SPROUTED SOYBEANS
(Average micrograms in the designated portion of one soybean sprouted 54 hours)

VITAMIN	FIRST SERIES		SECOND SERIES	
	HYPOCOTYL	COTYLEDON	HYPOCOTYL	COTYLEDON
Carotene	0.13	0.44	0.05	0.19
Thiamin	0.28	0.99	0.31	1.16
Riboflavin	0.14	0.55	0.16	0.46
Niacin	1.27	5.77	1.43	4.27
Ascorbic acid—Reduced	15.65	59.56	15.57	60.23
Ascorbic acid—Total	20.80	74.91	26.11	66.91

the dehydro form, average per soybean, in the soaked product before germination. Increases in both forms were marked after 24 hours; and the total had increased 5.9- and 5.4-fold in 48 hours in the two series, 7.1- and 5.6-fold in 54 hours, and 7.1- and 6.5-fold in 72 hours, for the two respective series. The concentration of reduced ascorbic acid fell in one series between 54 and 72 hours, although the total showed a slight increase during this period.

From the point of view of appearance and edibility, the 54-hour germination sample was superior to any of the others.

The distribution of the vitamins between the hypocotyls and the cotyledons of the sprouted soybeans after 54 hours of germination, in terms of average micrograms per bean are shown (table II and fig. 3). The cotyledons had almost four times as much carotene, more than three times as much thiamin, almost four times as much riboflavin, approximately four times as much niacin, and approximately three times as much total ascorbic acid as the hypocotyls, on individual soybean basis. The practice therefore of discarding the bean and retaining only the sprout involves a considerable loss in the vitamin content of the sprouted bean.

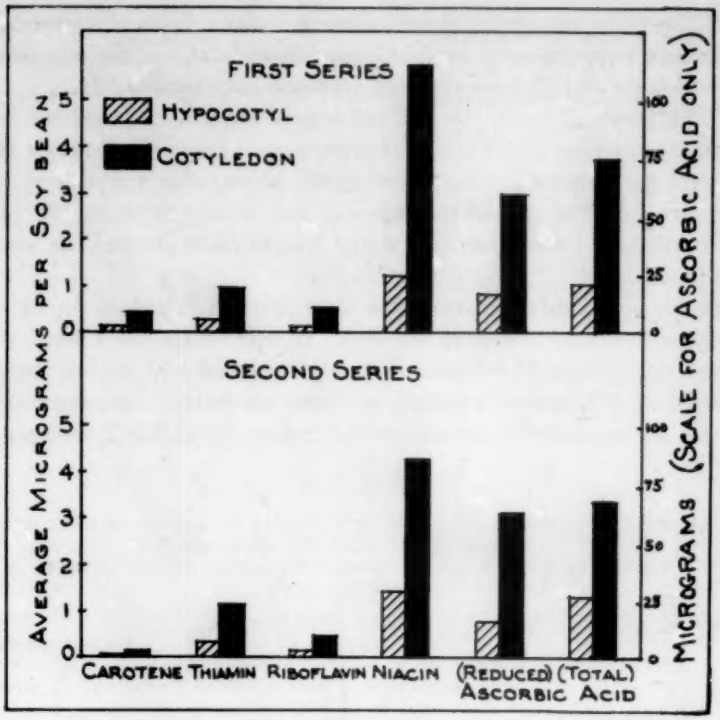

FIG. 3. Distribution of vitamins in the hypocotyl and cotyledon portions of sprouted Bansei soybeans after 54 hours of germination.

Summary

Assays for carotene, thiamin, riboflavin, niacin, and reduced and dehydro ascorbic acid were made on dry Bansei soybeans, and on the soybeans after controlled soaking and germination periods; the latter were 24, 48, 54, and 72 hours. The soaking and germination treatments were those selected after preliminary trials as giving a desirable edible product. Analyses for the same vitamins were made on the hypocotyl and cotyledon portions after 54 hours of germination.

The quantity of all vitamins studied except thiamin showed increases through 54 hours of germination, the period at which the product exhibited its optimum organoleptic properties. Thiamin showed alternate increases and decreases throughout the germination period investigated.

In the product of a 54-hour germination period, the cotyledons contained notably greater amounts of all vitamins for which tests were made. The process of sprouting soybeans, therefore, increases the nutrient value of the product. The entire bean should be eaten, since the cotyledons, which frequently are not retained, are a richer source of the vitamins for which tests are made than are the hypocotyls.

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EFFECTS OF NITROGEN ON THE NITROGENOUS FRACTIONS OF *ANANAS COMOSUS* (L.) MERR.[1]

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(WITH SIX FIGURES)

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Introduction

The scope of this study is to investigate the amounts of certain nitrogenous fractions in the tissues of *A. comosus* grown in solution cultures with high or low amounts of nitrogen supplied either as nitrate or ammonium salts; two previous papers also concerned with the same plants had as objectives the growth and content of certain nutrient elements (**35**), chlorophyll, and various carbohydrate fractions in the tissues (**36**).

Former studies (**30, 31, 32**) have indicated that the leaves of *A. comosus* grown in substrata containing ammonium were greener, broader and more succulent by comparison with those grown in nitrate. Also, the tissues of the former contained no nitrate but greater amounts of soluble organic-N than the latter. Such morphological and chemical differences serve as indexes of nitrogenous nutrition and may be utilized under field conditions for the adjustment of the nitrogenous requirements of plants.

Cultural and chemical methods

The composition of the culture solutions, employed for the growth of the experimental plants for one year, was reported in an earlier paper (**35**). The system employed for the nomenclature of the leaves, the technique for the sectioning of the various organs, the preparation of tissues and chemical methods for their analysis were reported in previous publications (**29, 30**).

Results

Total nitrogen

The data in table I, reporting the amounts of total nitrogen or its fractions as grams or as percentage of total nitrogen per plant or organ, show that the leaves comprised in the high-N cultures 85.46% and 91.63% and in the low-N cultures 68.80% and 69.14% of total nitrogen in the whole plant for the nitrate-N and ammonium-N series, respectively. Percentage of leaf weight for the same cultures, in the same table, was lower than percentage of nitrogen in the high-N cultures, but in the low-N cultures leaf weight percentage was higher than nitrogen percentage, indicating that the amount of nitrogen per unit of tissue was greater in the high-N than low-N cultures.

[1] Published with the approval of the Director as Technical Paper no. 171 of the Pineapple Research Institute, University of Hawaii.

TABLE I

TOTAL TISSUE CONTENT OF NITRATE-N, SOLUBLE ORGANIC-N, PROTEIN-N OR TOTAL-N PER PLANT OR ORGAN AND WEIGHTS FOR THE LATTER OF ONE-YEAR-OLD *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 OR 2.8 MILLIGRAMS OF N PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+

NITROGENOUS FRACTIONS OR PLANT WEIGHTS	PLANT ORGANS	NITRATE SERIES				AMMONIUM SERIES			
		HIGH-N		LOW-N		HIGH-N		LOW-N	
		gm.	%	*gm.*	%	*gm.*	%	*gm.*	%
Nitrate	Leaves	0.449	5.26	.....	.....	.....	.....	.....	.....
,,	Stem	0.164	1.93	.....	.....	.....	.....	.....	.....
,,	Roots	0.058	0.68	.....	.....	.....	.....	.....	.....
,,	Plant	0.671	7.87	.....	.....	.....	.....	.....	.....
Soluble Organic-N	Leaves	1.515	17.80	0.245	14.30	3.049	31.00	0.217	11.34
,, ,,	Stem	0.393	4.60	0.052	3.03	0.334	3.40	0.108	5.65
,, ,,	Roots	0.116	1.34	0.035	2.04	0.047	0.50	0.038	1.98
,, ,,	Plant	2.024	23.74	0.332	19.37	3.430	34.90	0.363	18.97
Protein-N	Leaves	5.313	62.40	0.935	54.50	5.958	60.63	1.107	57.80
,,	Stem	0.217	2.55	0.111	6.47	0.240	2.45	0.228	11.90
,,	Roots	0.293	3.44	0.337	19.66	0.198	2.02	0.217	11.33
,,	Plant	5.823	68.39	1.383	80.63	6.396	65.10	1.552	81.03
Total-N	Leaves	7.277	85.46	1.180	68.80	9.007	91.63	1.324	69.14
,,	Stem	0.774	9.08	0.163	9.50	0.574	5.85	0.336	17.55
,,	Roots	0.467	5.46	0.372	21.70	0.245	2.52	0.255	13.31
,,	Plant	8.518	100.00	1.715	100.00	9.826	100.00	1.915	100.00
Weights (gram)	Leaves	2952	81.00	1755	74.00	2660	86.60	2035	78.00
,, ,,	Stem	376	10.20	147	6.20	254	8.30	167	6.50
,, ,,	Roots	322	8.80	473	19.80	156	5.10	403	15.50
,, ,,	Plant	3650	100.00	2375	100.00	3070	100.00	2605	100.00

In the stem the ratio of nitrogen to total plant N was greater in the low-N than in the high-N cultures. Differences in nitrogen percentage of the stem between high-N and low-N cultures were greater in the ammonium-N than in the nitrate-N series, suggesting concentration effects, because stems as percentage of plant weights were also smaller in the latter cultures. In the roots nitrogen as percentage of total plant N was greater in the low-N than high-N cultures, and percentages of root weights were likewise greater in the low-N than high-N cultures, indicating positive correlation between amounts of nitrogen and root weights.

Total nitrogen values for the entire plant or leaves of the high-N cultures, reported as milligrams per gram of fresh tissue, were greater in the ammonium- than nitrate-N series (table II). Similar values for the low-N cultures were approximately the same in both series. Comparison of total nitrogen values between stem and leaves shows that they were greater for the former than latter organs in the low-N, but not in the high-N cultures. Total-N values for the roots were greater in the high-N than low-N cultures. The much greater total-N values in the ammonium-N than in the nitrate-N series indicate a greater rate of absorption of NH_4^+ than NO_3^-, which was also found in previous studies (**30**).

Soluble nitrogen

Soluble-N, comprising various compounds of inorganic-N and organic-N, was considerably greater for the high-N cultures in the ammonium than nitrate series (tables I, II; fig. 1). Differences in soluble-N between nitrate-N and ammonium-N series in the low-N cultures were very small excepting the stems. The leaves of the high-N cultures contained 17.80% and 31.00% of soluble-N for the NO_3 and NH_4 series, respectively (table I). But in the low-N cultures, similar values were 14.30% and 11.34% for the nitrate-N and ammonium-N series, respectively. Percentage of soluble-N in the stem was greater for the high-N than low-N cultures in the nitrate-N series, but in the ammonium-N series the percentage of soluble-N in the stem was greater for the low-N than the high-N cultures. In the roots percentage of soluble-N was higher in the low-N than high-N cultures.

Soluble-N gradients for the leaves of the cultures in the nitrate-N series had two maximal values, one in the basal (no. 1) section resulting from the sum of soluble organic-N and nitrate-N and another one in the terminal (nos. 4 or 5) sections from soluble organic-N alone (fig. 1). However, similar gradients for the high-N cultures in the ammonium-N series had only one maximal value in the terminal (nos. 4 or 5) sections, except in basal (no. 1) sections of the young (E) leaves, which resulted from soluble organic-N accumulations. But gradients for the low-N cultures in the ammonium-N series behaved irregularly, possibly because of the low nitrogen supply which limited accumulations of soluble organic-N.

Nitrate-N

Nitrate-N was found only in the high-N cultures of the nitrate-N series

TABLE II

MEAN VALUES OF NITRATE-N, SOLUBLE ORGANIC-N, PROTEIN-N AND TOTAL-N AS MILLIGRAMS PER GRAM OF FRESH TISSUE OF *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+

PLANT AND ORGANS	NITRATE SERIES							AMMONIUM SERIES					
	HIGH-N				LOW-N			HIGH-N			LOW-N		
	TOTAL N	NO_3^- N	SOLUBLE ORG-N	PROTEIN-N	TOTAL N	SOLUBLE ORG-N	PROTEIN-N	TOTAL N	SOLUBLE ORG-N	PROTEIN-N	TOTAL N	SOLUBLE ORG-N	PROTEIN-N
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Leaves	2.470	0.152	0.518	1.800	0.673	0.140	0.533	3.385	1.145	2.240	0.651	0.107	0.544
Stem	2.060	0.437	1.045	0.578	1.110	0.354	0.756	2.260	1.315	0.945	2.012	0.647	1.365
Roots	1.450	0.018	0.360	0.910	0.787	0.075	0.712	1.572	0.302	1.270	0.632	0.094	0.538
Plant	2.335	0.184	0.555	1.596	0.723	0.140	0.583	3.196	1.116	2.080	0.734	0.139	0.595

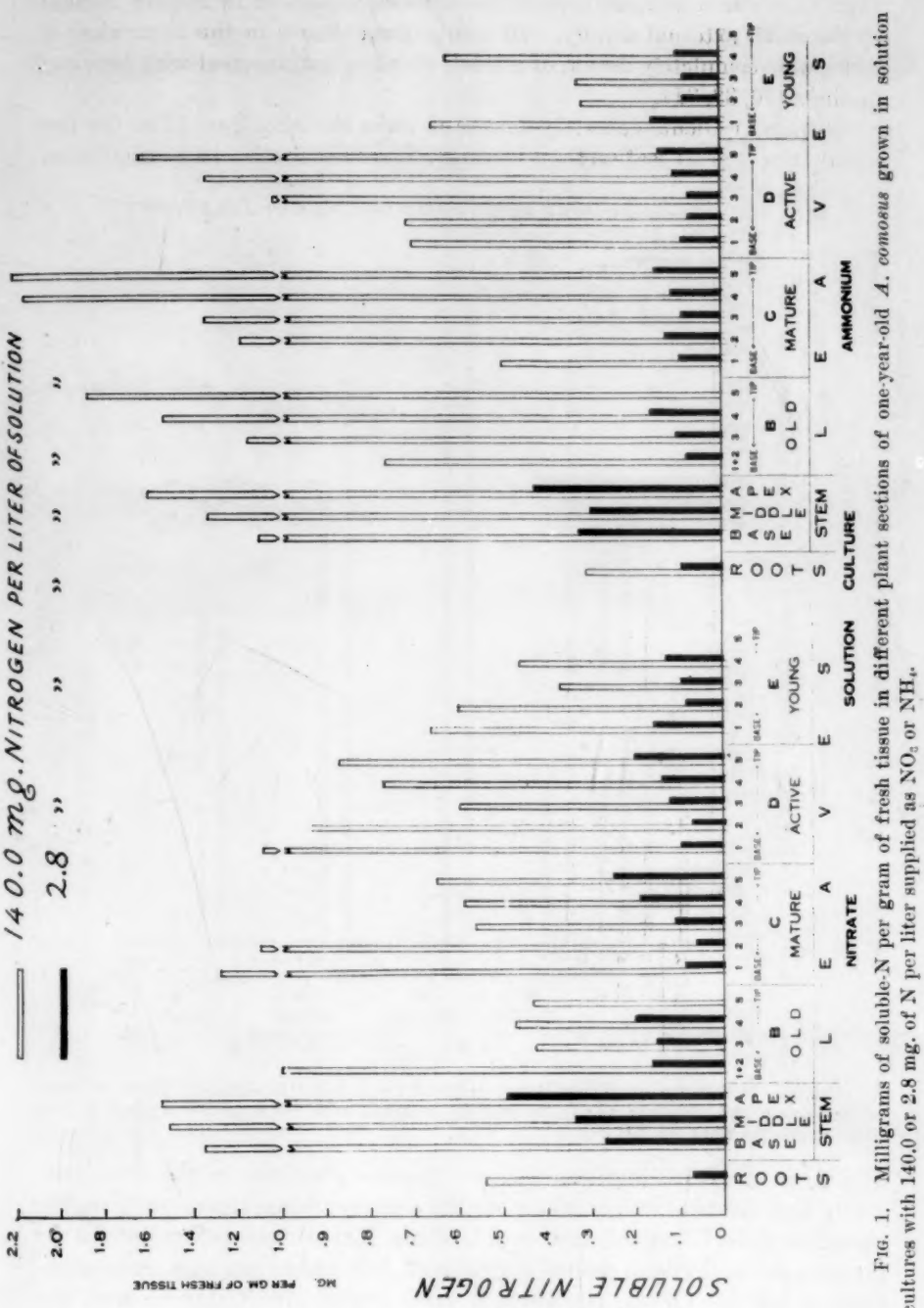

FIG. 1. Milligrams of soluble-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

(fig. 2). The low-N cultures in the same series lacked in nitrate because of the small external supply. Of course the cultures in the ammonium-N series were completely devoid of nitrate, which is in agreement with previous findings (**30, 33, 34**).

Nitrate gradients generally decreased from the basal (no. 1) to the terminal (nos. 4 or 5) leaf sections because of rapid reduction and assimilation

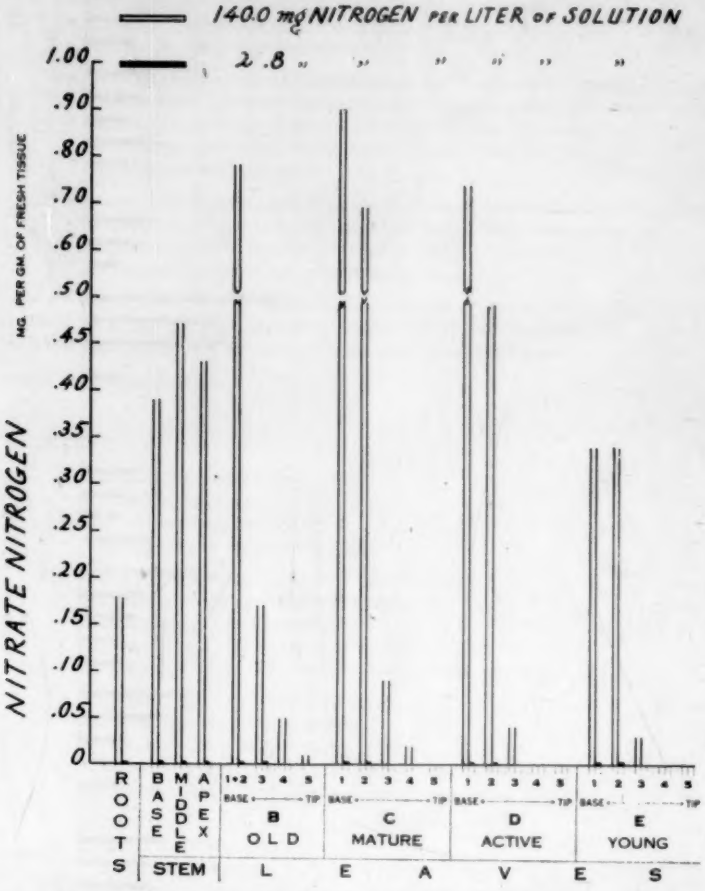

FIG. 2. Milligrams of nitrate-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

of NO_3 in the chlorophyllous tissues. Similar gradients in the stem generally had maximal values in the medial and may have occasionally in the apical sections. Unpublished data indicate that nitrate accumulations in the non-chlorophyllous tissues may attain, but seldom surpass, concentrations as high as 1.5 mg. per gram of fresh tissue, presumably because the rate of nitrate absorption is dependent on the amount of available utilizable energy at the absorbing regions of the root. Such regions in *A. comosus*

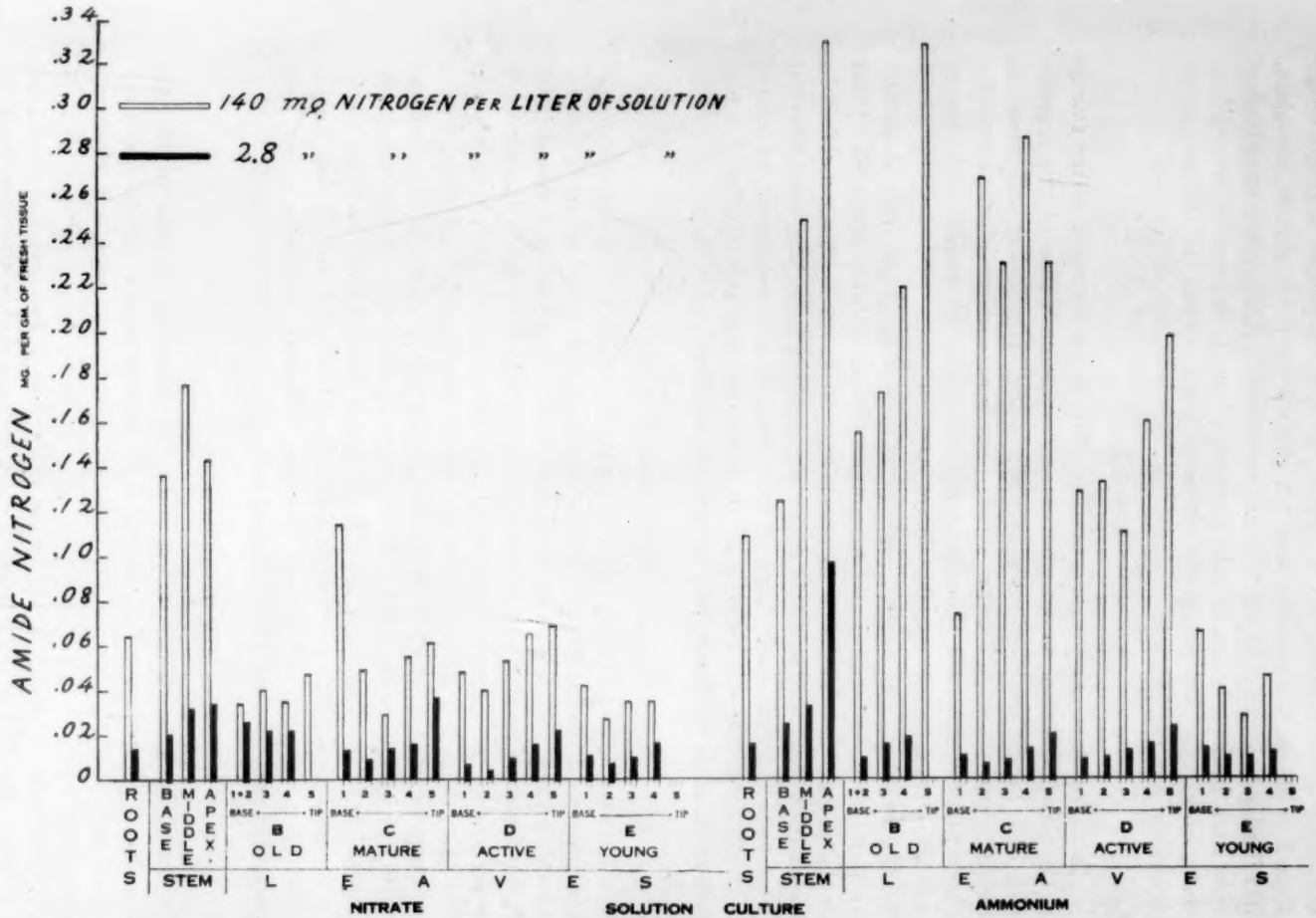

FIG. 3. Milligrams of amide-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

beginning approximately 0.5 cm. from the root tip proper and extending from 1 to 10 cm. towards the base are characterized by whitish, succulent, non-lignified tissues with or without root hairs. Also, other unpublished data have indicated that nitrate concentrations in the non-chlorophyllous leaf regions may depend on the ratios of plant volume to the surface of absorbing root regions.

Amide-N

In agreement with previous studies (**30**), amide nitrogen in the tissues of the high-N cultures, was greater in the ammonium-N than nitrate-N series (fig. 3). In the low-N cultures only slight and insignificant differences in amide-N were observed between series. The basal sections of the young (E) leaves and the apical of the stem composed mostly of meristematic tissues contained considerable amounts of amide-N.

The results might suggest direct synthesis of amide-N from oxalacetic acid or α-ketoglutaric acid and ammonia in the ammonium series (5, 8) but in the nitrate-N series similar synthesis may be doubted. Some amide-N in the ammonium-N and probably all in the nitrate-N series may have resulted from protein breakdown. Lack of knowledge of any mechanism associated with the reduction and assimilation of nitrate renders any intelligent explanation of amide synthesis from nitrates impossible.

Mono-amino-N

Mono-amino-N for the high-N cultures was greater in the ammonium-N than nitrate-N series (fig. 4). The differences in mono-amino-N for the low-N cultures between series were small. The gradient of mono-amino-N in the basal sections of the young (E) leaves was reversed with respect to other leaves. The greater mono-amino-N content of the high-N cultures in the ammonium-N than nitrate-N series was presumably associated with a much greater intake by plants of NH_4^+ than NO_3^-, which is in agreement with previous findings (**30**). Mono-amino-N gradients in the leaves increased from the basal (no. 1) to the terminal (nos. 4 or 5) sections except in the young (E) leaves where the content of the basal (no. 1) was greater than of the transitional (no. 2) sections. Similar gradients in the stem increased from the basal to the apical sections.

Basic-N

Basic-N in the tissues of the high-N cultures was greater in the ammonium-N than nitrate-N series (fig. 5). Similar values for the low-N cultures were slightly greater for some sections in the nitrate-N than ammonium-N series.

Gradients of basic-N in the leaves of the high-N cultures increased from the basal (no. 1) to the terminal (no. 5) sections and in the stem from the basal to the apical sections except the medial in the nitrate-N series. Basic-N levels in the high-N cultures being greater for the ammonium-N than

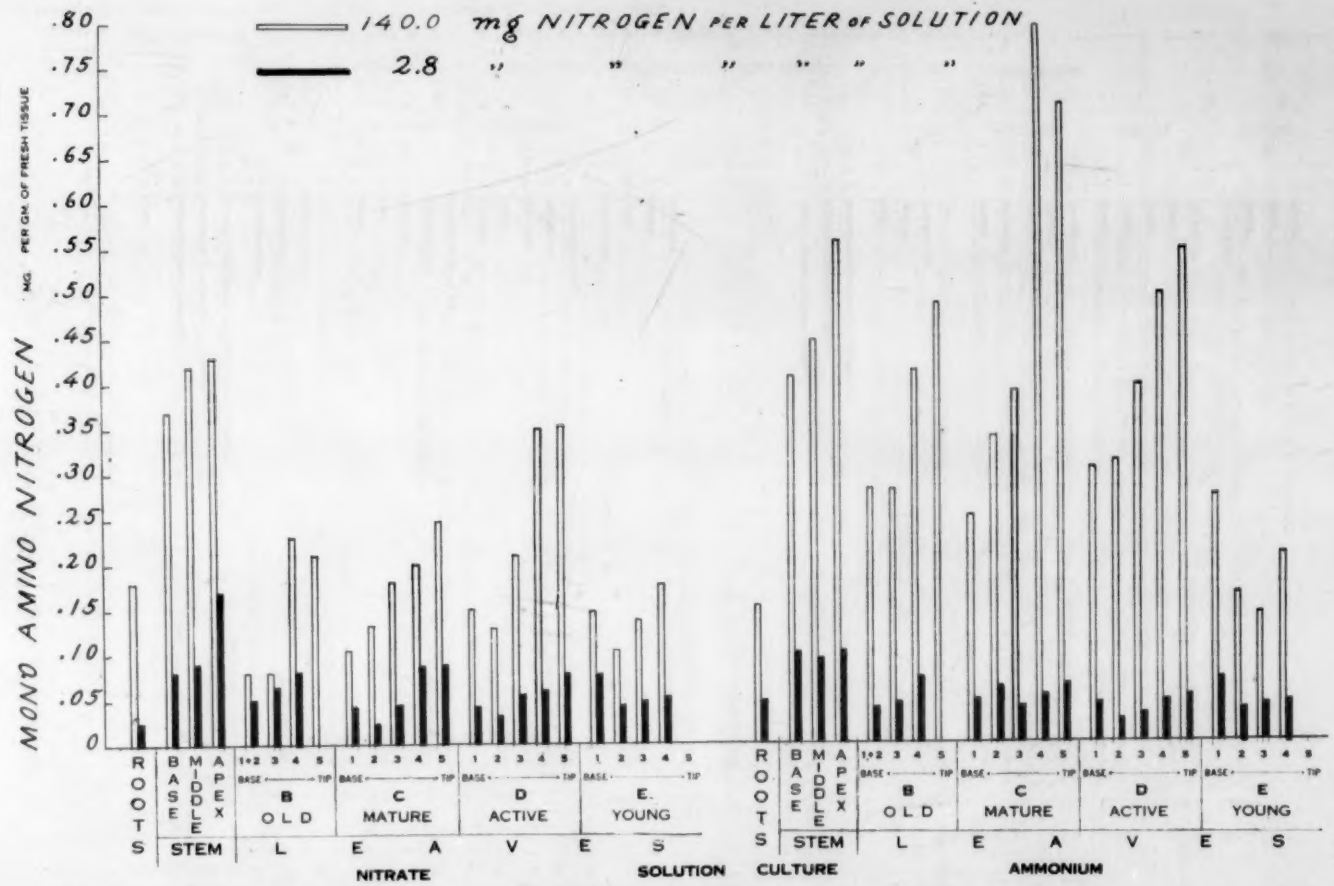

FIG 4. Milligrams of mono-amino-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

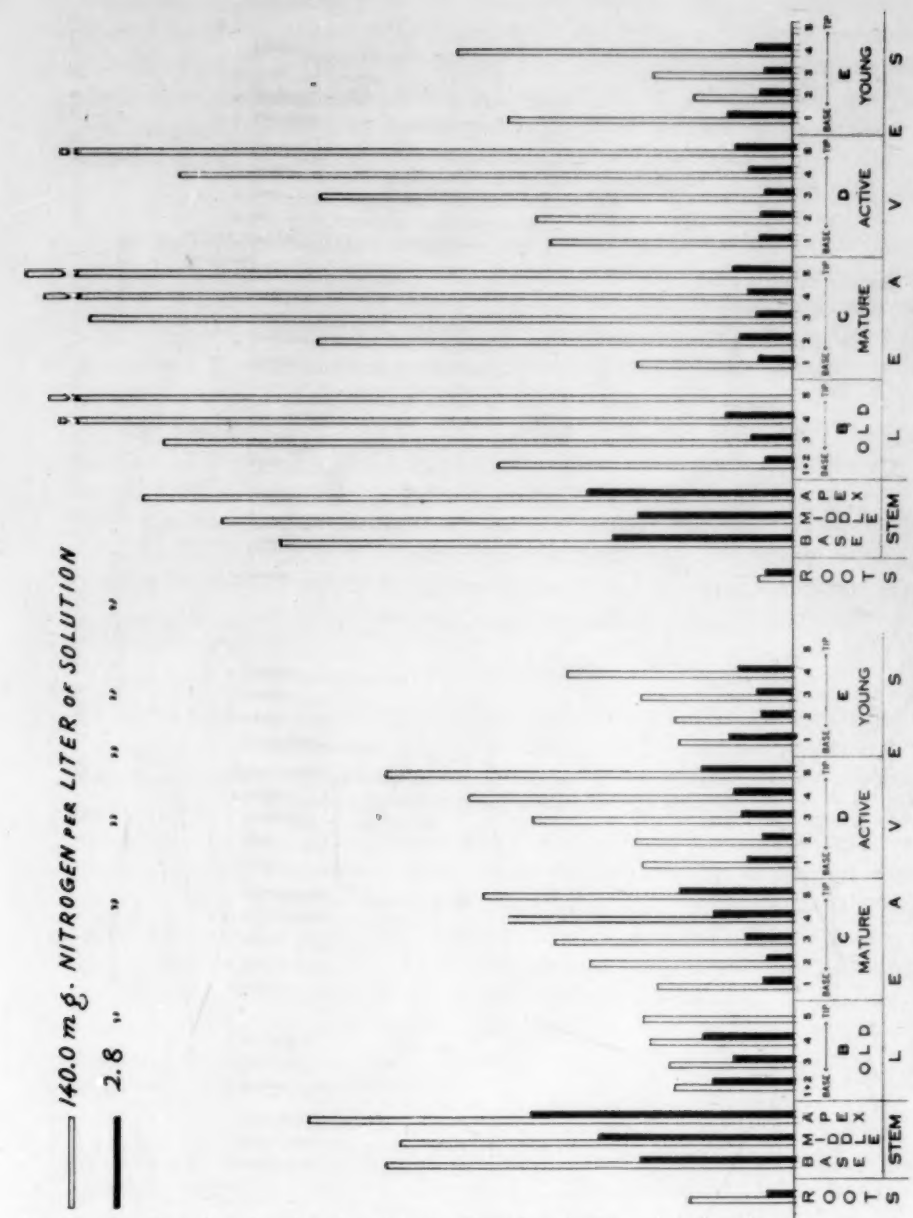

FIG. 5. Milligrams of basic-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

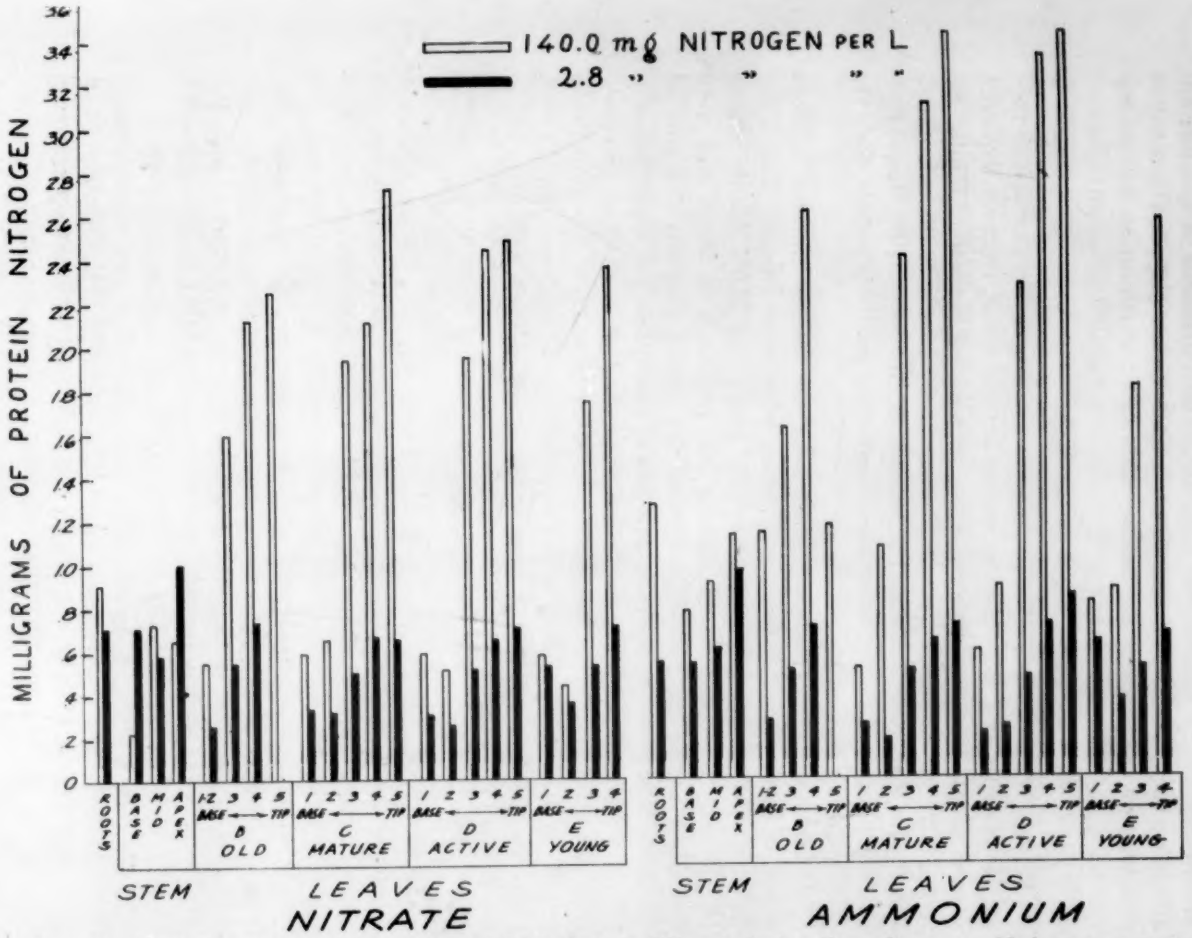

FIG. 6. Milligrams of protein-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

nitrate-N series cannot be attributed to direct synthesis from ammonium with some carboxylic acids and might have resulted from protein breakdown because experimental evidence on basic amino acid synthesis is lacking; but according to MOTHES (**17**), and KLEIN and TAÜBOCK (**12**), the content of free base in the sap may depend on the rate of reutilization for protein synthesis.

PROTEIN-N

Protein-N for the high-N cultures was greater in the ammonium than nitrate-N series (fig. 6). Similar values for the low-N cultures, however, showed slight differences between series, presumably because of limited external nitrogen supplies. Protein-N accumulated mostly in the chlorophyllous tissues and correlated with the amounts of chlorophyll; the non-chlorophyllous tissues of the leaves or stem containing smaller amounts than the chlorophyllous tissues.

Differences in the protein-N content of the high-N cultures between series, in favor of the ammonium-N series, were approximately 31.0% for the combined mature (C) and active (D) leaves and 18.0% for the young (E) leaves. Such differences indicate that protein-N in leaves may vary depending on the chlorophyll content of the tissues, amounts and kind of inorganic-N supplied to the roots and rate of absorption and assimilation of same. Protein-N gradients in the leaves of the high-N cultures increased from the basal (no. 1) in the ammonium-N series or from the transitional (no. 2) in the nitrate-N series to the terminal (no. 5) sections. In the stem gradients increasing from the basal to the apical sections were observed in the ammonium-N series.

Discussion

MCKEE (**16**), NIGHTINGALE (**20, 21**), CHIBNALL (**8**), PETRIE (**25**) and WOOD (**45**) have reviewed the various phases of nitrogen metabolism in green plants. Studies by the authors (**30, 33, 34**) showed that NH_4^+ is assimilated by the root tissues of *A. comosus* almost as soon as it is absorbed and converted to amide-N, amino-N and protein-N, whereas NO_3^- is translocated from the roots to the non-chlorophyllous regions of the leaves in the original state and assimilated in the chlorophyllous regions. VICKERY *et al.* (**40**) employing N^{15} in ammonium salts as a tracer, and in other experiments (**41**) found that tobacco plants also assimilated NH_4^+ in a similar manner as *A. comosus*. BURSTRÖM (**6, 7**) presented evidence that stored nitrate in the leaves of wheat plants is assimilated only in light and that with increasing light intensity there is an increase in the assimilation of nitrate, apparent assimilation of CO_2, and formation of sugar; whereas, in darkness, nitrate accumulated within the leaves is not assimilated and sugar is oxidized to CO_2, which is quantitatively given off as respiration CO_2. The same author (**6**) suggested that a different mechanism operates for the assimilation of nitrates by roots. Also the experiments of BLACKMAN and TEMPLEMAN (**2**) suggested that light is necessary for nitrate assimilation. However, nitrate

assimilation in light according to NIGHTINGALE and SCHERMERHORN (**19**) may be characteristic of certain species of plants but in others nitrate reduction may be effected in darkness and in light mainly by the fine rootlets. Energy utilization, possibly derived from carbohydrates, is prerequisite for the absorption of nitrate and other anions from substrata, according to LUNDEGARDH (**14**), GILBERT and SHIVE (**9**), and HAMNER (**11**).

Experimental evidence for the conversion mechanism of NH_4^+ to amide-N and amino-N has been obtained (**5**) but not for NO_3^-. SCHULZE (**28**) expressed the opinion that amides arise by the reaction of ammonia, derived from amino acids of seed proteins, with nitrogen-free substances, while VICKERY *et al.* (**42**) believe that the precursors most probable for amino acids are the corresponding α-keto acids. CHIBNALL (**8**) suggested the citric acid cycle of KREBS and JOHNSON (**13**) providing a chemical mechanism for the formation of oxalacetic or α-ketoglutaric acids which may react with ammonia to form by enzymic activity aspartic, or glutamic acids or their respective amides, asparagine or glutamine. BRAUNSTEIN and KRITZMANN (**5**) demonstrated that amino nitrogen may be transferred to keto acids by transamination and ALBAUM and COHEN (**1**) showed that transamination between glutamic and oxalacetic acid occurs in oat seedlings. MOTHES (**18**) claimed amino acids may be formed enzymically in tissues either by combination of ammonia with carboxylic acids or by protein hydrolysis. BORSOOK and HUFFMAN (**4**) effected synthesis of aspartic acid from fumaric acid and ammonia, and of alanine from pyruvic acid and ammonia. VIRTANEN and LAINE (**43**) claimed that aspartic acid is the first amino acid formed in plants from which other amino acids may be produced by transference of the amino group to α-keto acids, but acid amides, *e.g.*, glutamine or asparagine, cannot take part in transamination directly except after the splitting of the acid amide group. RAUTANEN (**26**), having studied transamination in green plants, claimed that in systems in which l-glutamic acid or α-ketoglutaric acid acted as the dicarboxylic acid component than l-aspartic acid or oxalacetic acid, transamination was quicker. Also, because of the formation of alanine and certain aliphatic amino acids through transamination in plants, l (−) aspartic acid + pyruvic acid $\rightleftharpoons$ Oxalacetic acid + l (+) alanine, he suggests that most amino acids can be regarded as derivatives of alanine.

Protein metabolism, in animals in nitrogen equilibrium, is, according to BORSOOK and KEIGHLEY (**3**), a process of continual breakdown of intracellular protein even when abundant quantities of amino acids are obtained from the diet, and as a consequence a corresponding quantity of amino acids is synthesized into tissue proteins and peptides. The literature pertaining to protein metabolism in plants indicates that a mechanism may be operating for the protein-N $\rightleftharpoons$ soluble organic-N reaction somewhat similar to that in animals as postulated by BORSOOK and KEIGHLEY (**3**) or SCHOENHEIMER *et al.* (**27**).

The data in table III indicating ratios of protein-N to soluble organic-N for the high-N and low-N cultures in the nitrate-N series suggest that there

TABLE III

MEAN VALUES OF TOTAL ORGANIC-N, PROTEIN-N AND SOLUBLE ORGANIC-N AS MILLIGRAM WEIGHT OF ENTIRE SECTIONS AND RATIOS OF PROTEIN-N (P. N.) TO SOLUBLE ORGANIC-N (S. O. N.) FOR THE COMBINED SECTIONS AS INDICATED OF *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+

PLANT SECTIONS	NITRATE-N								AMMONIUM-N							
	HIGH-N				LOW-N				HIGH-N				LOW-N			
	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S. O. N.	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S. O. N.	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S. O. N.	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S. O. N.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	
Leaves:																
Old (B)																
1 + 2	78.0	22.2	55.8	0.395	13.5	8.1	5.4	1.50	74.1	44.3	29.8	1.49	16.3	12.5	3.8	3.29
3 – 5	684.0	562.0	122.0	4.600	104.5	81.6	22.9	3.57	727.0	400.5	326.5	1.23	156.8	127.1	29.7	4.28
Mature (C)																
1 + 2	278.0	89.7	188.3	0.477	23.6	19.3	4.3	4.50	171.3	82.6	88.7	0.93	22.0	14.0	8.0	1.75
3 – 5	2294.0	1838.0	456.0	4.040	280.5	214.2	66.3	3.24	2650.0	1602.0	1048.0	1.53	288.0	242.0	46.0	5.26
Active (D)																
1 + 2	345.6	110.1	235.5	0.418	44.3	34.0	10.3	3.30	285.0	144.5	140.5	1.03	45.8	33.5	12.3	2.72
3 – 5	2497.0	1924.0	573.0	3.355	382.3	307.5	74.8	4.10	3440.0	2401.0	1039.0	2.31	471.4	406.2	65.2	6.23
Young (E)																
1 + 2	408.0	109.2	298.8	0.365	146.2	114.2	32.0	3.57	540.0	342.5	197.5	1.73	163.2	131.1	32.1	4.08
3 – 4	785.0	652.0	133.0	4.900	186.0	156.7	29.3	5.37	1042.0	859.8	182.2	4.72	175.4	151.6	23.8	6.37
Total leaves:																
Av. 1 + 2	1109.6	331.2	778.4	0.412	227.6	175.6	52.0	3.38	1070.4	613.9	456.5	1.35	247.3	191.1	56.2	3.40
3 – 5	6260.0	4976.0	1284.0	3.865	953.3	760.0	193.3	3.93	7859.0	5263.3	2595.7	2.03	1091.6	926.9	164.7	5.63
Stem:																
Base + Mid. + Apex	872.6	216.6	656.0	0.330	161.0	109.4	51.6	2.12	581.0	239.0	342.0	0.70	173.1	115.3	57.8	1.99
Roots	532.0	294.0	238.0	1.236	372.0	337.0	35.0	9.63	245.0	198.0	47.0	4.22	255.0	217.2	37.8	5.75

TABLE IV

PERCENTAGE OF PROTEIN-N AND SOLUBLE ORGANIC-N AS TOTAL ORGANIC-N IN THE COMBINED SECTIONS, REPORTED IN TABLE III, OF ONE-YEAR-OLD *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+

PLANT SECTIONS		NITRATE-N				AMMONIUM-N			
		HIGH-N		LOW-N		HIGH-N		LOW-N	
		PROTEIN-N	SOLUBLE ORG-N	PROTEIN-N	SOLUBLE ORG-N	PROTEIN-N	SOLUBLE ORG-N	PROTEIN-N	SOLUBLE ORG-N
		%	%	%	%	%	%	%	%
Leaves:									
Old	(B) 1+2	28.5	71.5	60.0	40.0	59.8	40.2	76.7	23.3
	3–5	82.0	18.0	78.0	22.0	55.0	45.0	81.0	19.0
Mature	(C) 1+2	32.0	68.0	81.8	18.2	48.2	51.8	63.6	36.4
	3–5	80.0	20.0	76.4	23.6	60.4	39.6	84.0	16.0
Active	(D) 1+2	31.9	68.1	76.8	23.2	50.7	49.3	73.1	26.9
	3–5	77.0	23.0	80.3	19.7	69.8	30.2	86.0	14.0
Young	(E) 1+2	26.7	73.3	78.2	21.8	63.4	36.6	80.4	19.6
	3–4	83.0	17.0	84.4	15.6	82.6	17.4	86.5	13.5
Total leaves:									
Average	1+2	29.0	71.0	77.3	22.7	57.4	42.6	77.3	22.7
	3–5	79.5	20.5	79.8	20.2	67.0	33.0	85.0	15.0
Stem:									
Base + Mid. + Apex		24.9	75.1	68.0	32.0	41.2	58.8	66.4	33.6
Roots		55.3	44.7	90.5	9.5	80.8	19.2	85.2	14.8

is possibly a continuous breakdown of proteins to amino acids or conversion of the latter to the former. If we assume that the ultimate fate of nitrogen in plant metabolism is protein synthesis and that the reaction, soluble organic-N → protein-N is irreversible, then the presence of any soluble organic nitrogen fraction in substantial amounts in the tissues should constitute an anomaly except in cultures supplied with great amounts of inorganic nitrogen and operating under conditions retarding the conversion of soluble organic-N to protein-N. Even the tissues of the low-N cultures, grown under conditions of partial nitrogen starvation, contained appreciable amounts of soluble organic nitrogen (table III) which, calculated as percentage of total organic nitrogen (table IV), was almost as high as that of the high-N cultures. The ratios of protein-N to soluble organic-N in the

TABLE V

CORRELATION COEFFICIENTS (r) AND SIGNIFICANCE (t) AT 5% LEVELS OF PROTEIN-N TO VARIOUS FRACTIONS OF SOLUBLE ORGANIC-N IN THE TISSUES OF DIFFERENT LEAF SECTIONS OF ONE-YEAR-OLD *Ananas comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER OF SOLUTION SUPPLIED AS NITRATE OR AMMONIUM SALTS

ITEMS OF CORRELATION	NITRATE SERIES				AMMONIUM SERIES			
	HIGH-N		LOW-N		HIGH-N		LOW-N	
	r	t	r	t	r	t	r	t
Soluble organic-N	−0.78	*5.00*	0.59	*2.83*	0.66	*3.52*	0.52	*2.37*
Amide-N	0.31	1.30	0.43	1.85	0.33	1.40	0.46	2.00
Mono-amino-N	* 0.84	* *4.85*	* 0.56	* 1.90	0.70	*3.92*	0.54	*2.47*
Basic-N	0.72	*4.17*	0.64	*3.23*	0.55	*2.62*	0.44	1.90
For statistical significance at:								
P .05	.....	2.12	.....	2.13	.....	2.12	.....	2.13
* P .05	.....	* 2.26	.....	* 2.31	.....	.....	.....	.....

* Number of samples, 11, because of omission of basal sections; all other samples were 18.

chlorophyllous sections (nos. 3, 4, 5) of the high-N cultures were smaller in the ammonium-N than in the nitrate-N series because of much greater accumulations of soluble organic-N in the former series presumably resulting either from direct synthesis by combination of ammonia with carboxylic acids or from interference in the rate of conversion of soluble organic-N to protein-N by the toxic effects of high Cl^- concentrations (350 mg. per liter of nutrient solution).

In general protein-N as percentage of total organic-N in the proximal (nos. 1–2) sections was greater in the low-N than in the high-N cultures (table IV), presumably either because of a lower rate of protein hydrolysis in the former than latter cultures or because the rate of synthesis of soluble organic-N is dependent on the external supplies of inorganic nitrogen. Percentage of protein-N or soluble organic-N in the chlorophyllous sections

of the young (E) leaves varied slightly between different cultures because the tissues were chronologically young and insufficient time had elapsed for the accumulation of soluble organic-N.

The greater values of the ratios of protein-N to soluble organic-N (table III), in the chlorophyllous sections (nos. 3, 4, 5) than in the non-chlorophyllous or partly chlorophyllous sections (no. 1, 2) should be attributed to the greater chlorophyll content of the former sections, which was found to correlate with the protein content of the tissues. Total nitrogen in the tissues increased in general with greater amounts of inorganic nitrogen in the nutrient solutions and *vice versa* (figs. 1–6).

Protein-N correlated directly with most fractions of soluble organic-N in the tissues (table V). The data indicate that correlation between protein-N and amide-N was lacking in all cultures. However, correlation between protein and mono-amino-N or basic-N was highly significant for the high-N cultures. Similar correlation was found in the low-N cultures except for mono-amino-N and basic-N in the nitrate-N and ammonium-N series, respectively. Moreover, in order to obtain definite correlation for mono-amino-N in the nitrate series it was necessary to exclude the non-chlorophyllous (no. 1) and partly chlorophyllous or transitional (no. 2) sections because the content of mono-amino-N in relation to protein-N was too high. It is assumed that a portion of this mono-amino-N was translocated to the proximal (nos. 1 and 2) sections of the leaves from the distal chlorophyllous (nos. 3, 4, 5) sections where it was derived by reduction and subsequent assimilation from nitrate-N. A similar condition did not obtain for the cultures in the ammonium series possibly because ammonium ions were assimilated by the root tissues as soon as they were absorbed and then translocated as fractions of soluble organic nitrogen. Therefore, correlation of protein-N to mono-amino-N and basic-N might indicate a continuous process of protein breakdown to mono-amino-N and basic-N and synthesis of the former by the reverse process.

Wood and Petrie (**43**) also found that definite relations existed between synthesis of protein in plant tissues and content of amino acids, ammonia and water. However, the rate of protein formation in tissues may be retarded by a decreased rate of synthesis of cystine, according to Petrie and Wood (**23, 24**) or of methionine, according to Lugg and Weller (**15**). In order to explain the regulation of protein decomposition and synthesis in plants Paech (**22**) formulated the "mass action" hypothesis which postulates that a single mass law reaction exists between proteins, on the one hand, and a carbohydrate and ammonia, on the other. A somewhat similar view is shared by Gregory (**10**) who stated that if protein synthesis were the reverse of proteolysis, the direction of the reaction would be determined by mass action and the velocities of the reactions concerned. Variation in velocity of the reactions concerned would lead to accumulation; thus, if the synthetic route to protein were rapid, protein would tend to accumulate, and by mass action increase the breakdown to amino acid. If these amino

acids, before being again used in protein synthesis, were deaminated, and if this particular reaction were the slowest in the cycle, then amino acids would accumulate. However, the great complexity of factors already known (**15, 25**) to be associated with protein synthesis or breakdown weakens PAECH's hypothesis with respect to the effects of mass law action.

The direction and velocity of the reaction soluble organic-N $\rightleftharpoons$ protein-N for different plant sections (tables III and IV), which apparently were different for the chlorophyllous than non-chlorophyllous sections of the leaves, were probably affected by factors associated with the physiological functions of the tissues concerned in a somewhat similar manner as claimed by SCHOENHEIMER *et al.* (**27**) for animal tissues; the authors observed by means of N^{15}, employed as a tracer, that all the proteins of the body are continually undergoing synthesis and breakdown, the process being more rapid in some organs as the liver and kidneys than in others, as the erythrocytes. In the chlorophyllous sections (nos. 3–5) where considerable protein is a constituent of the chloroplastic stroma, protein-N values were generally greater than in sections 1 and 2, the former lacking in and the latter containing small amounts of chlorophyll. Protein amounts in the chlorophyllous sections did not vary as much for similar cultures between different series as did soluble organic-N. These results suggest that the amounts of soluble organic-N, attributable to protein breakdown, may be in the range of the ratios of protein-N to soluble organic-N, indicated in table III, for the low-N cultures because the greater soluble organic-N content of the high-N cultures might possibly contain small portions synthesized directly from ammonia and keto acids and not derived from protein breakdown.

The smaller ratios of protein-N to soluble organic-N for the non-chlorophyllous or partly chlorophyllous sections (nos. 1 and 2) than for the chlorophyllous (nos. 3–5) may be attributed to differences in physiological functions. The former composed of meristematic tissues are concerned with growth processes, and the latter, concerned with photosynthesis, require greater amounts of protein for the proteinaceous stroma of the chloroplasts. Another possibility for the lower ratios of protein-N to soluble organic-N in the non-chlorophyllous than chlorophyllous sections may be the greater growth activity of the former which, dependent on cell division, would tend to divide the protein content whereas in the chlorophyllous mature tissues the protein content of the cells would increase by successive depositions.

The association of lower ratio values of protein-N to soluble organic-N with tissues undergoing growth activity is in agreement with the findings of TRAUB (**39**) and THOMAS (**38**) who observed that apple twigs, during the plant's most active period of growth, contained higher amounts of amino-N but lower amounts of protein-N and total carbohydrates. Therefore, factors which admittedly affect the velocity of the reaction soluble organic-N $\rightleftharpoons$ protein-N are: (a) stage of plant growth, (b) amounts and kinds of inorganic nitrogen absorbed by roots, (c) conditions affecting carbohydrate synthesis, (d) water and concentrations of essential and accessory nutrient elements, and (e) oxygen tension (**37**).

Summary

The effects of high *vs.* low nitrogen (140.0 *vs.* 2.8 mg. per liter of nutrient solution) supplied either as NO_3^- or NH_4^+ caused changes in the amounts of the various nitrogenous fractions of the tissues of one-year-old *A. comosus*. The plants of the high-N cultures absorbed approximately five times more nitrogen than of the low-N cultures, although the nitrogen content of the nutrient solutions was fifty times greater for the former than latter cultures.

Total-N and protein-N were from three to four times, and soluble-N was from four to eight times greater in the high-N than low-N cultures. Soluble organic-N was higher in the chlorophyllous than non-chlorophyllous regions of the leaves. Soluble organic-N fractions such as amide, mono-amino and basic were higher in the ammonium than nitrate series. Leaf protein-N was higher in the terminal chlorophyllous than in the basal non-chlorophyllous regions of the leaves.

Protein-N correlated with few exceptions to mono-amino-N and basic-N. The results suggest a continuous formation of amino nitrogen from proteins undergoing normal hydrolysis in the cell or synthesis of proteins by condensation of amino acids in cultures supplied with either adequate or subadequate supplies of inorganic nitrogen. Excessive supplies of inorganic nitrogen either as NH_4^+ or NO_3^- may cause accumulations of soluble organic-N fractions produced by enzymatic synthesis from ammonia with carboxylic acids and similar to those presumably released from protein breakdown.

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THE USE OF 2,3,5-TRIPHENYL-TETRAZOLIUMCHLORIDE AS A MEASURE OF SEED GERMINABILITY[1]

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(WITH ONE FIGURE)

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Seed analysts have long been interested in methods of measuring the germinability of seeds without the necessity of a routine germination test, particularly when dealing with dormant seeds or with seeds requiring a long period for the completion of a test. Even with seeds that can be tested in a week or 10 days it is often desirable to know within a day the general condition of a seed lot. This is especially important in the fall season when it is necessary to know whether or not an early killing frost has caused damage to the seed crop of corn, sorghum, and soybeans.

For dormant seeds, especially the peach, apple, pine, Douglas fir, plum, hawthorn, European mountain ash, witch hazel, and *Rhodotypos kerrioides*, normally requiring a long period of after-ripening, it has been shown by Barton (**1**), Flemion (**6, 7, 8, 9**) and others that by excising the embryos and placing them on moist filter paper in Petri dishes at room temperature it is possible to determine their vitality within a period of 5 to 10 days. Crocker and Harrington (**2**), Davis (**3**), and Leggatt (**13**) have published data indicating that the determination of the catalase ratio of dry and germinating seeds may serve as a measure of seed viability. Mar (**14**) has shown that the amylase activity of soaked oat seed is definitely correlated with germinability. Lakon (**10**) and others (**4, 5, 15**) have published data on the use of selenium and tellurium salts in solution as a means of determining the viability of a given seed lot by color reaction of the embryo. Lakon (**11, 12**) in 1942 compared his results by regular germination test of seeds of oats, barley, wheat, rye, and corn with those obtained by placing the cut seeds in a solution of 2,3-diphenyl-5-methyl-tetrazoliumchloride or 2,3,5-triphenyl-tetrazoliumchloride. The resultant staining of the viable embryos correlated well with his germination results.

Tetrazoliumchloride is colorless but forms carmine red formazans upon reduction. The salt is thus an oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is presumably an indication of the presence of active respiratory processes in which hydrogen radicals are transferred to the tetrazoliumchloride.

In 1945 a supply of 2,3,5-triphenyl-tetrazoliumchloride was obtained by officers of the Chemical Warfare Service in Germany, and Dr. Georg Lakon provided information about the testing program developed by him in which this material was used. Copies of publications, unobtainable in the past,

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were received. With the assistance of the junior authors a series of tests was undertaken with seeds of corn (*Zea mays*), barley, (*Hordeum vulgare*), oats (*Avena sativa*), wheat (*Triticum aestivum*), buckwheat, (*Fagopyron esculentum*), cotton (*Gossypium herbaceum*), pea (*Pisum sativum*), rice (*Oryza sativa*), sorgo (*Holcus sorghum*), soybeans (*Soya max*), vetch (*Vicia sativa*), and Bahia grass (*Paspalum notatum*).

Procedure

The use of the tetrazoliumchloride test for corn, barley, oats, rye, and wheat as described by LAKON (**11, 12**) consisted of soaking seeds to be tested in water for several hours to permit absorption of water and initiation of germination processes. Following the soaking period each seed was bisected longitudinally through the center of the embryo so that each half had a part of the plumule and the radicle. One half of each seed was then placed in a Petri dish and a 1% solution of tetrazoliumchloride poured over the cut seeds until they were completely immersed. The dish was kept for 2 to 10 hours in a dark cabinet at room temperature. At the end of the immersion period each half seed was examined to determine the degree to which the parts of the embryo were stained carmine red. LAKON (**11**) stated distinct staining of the vital parts of the embryo was evidence of ability to produce a normal seedling. For cereal seeds he concluded that if both the embryo and the scutellum stained carmine red, the seed would be strongly viable. His criterion for seeds of corn (**12**) was unusual, for he did not consider the primary root in corn absolutely essential to the production of a normal seedling, especially if secondary roots were produced. His view coincides with that of PORTER (**16**) and also with a report of the Committee on Standardized Tests of the Association of Official Seed Analysts (**17**). LAKON (**12**) observed that in the region commonly referred to as mesocotyl, buds were produced on the sides of the region between the plumule and radicle and that in viable seeds these structures stained carmine red, developing into secondary roots. He concluded, therefore, that viable seeds of corn were those which have stained: (1) the entire embryo and scutellum, (2) all parts of the embryo and scutellum including the lateral buds in the mesocotyl region, but not the radicle, or (3) the entire embryo, but not the scutellum; he doubted that all seeds of this group were included among the normal seedlings. In general, it was his belief that a seed possessed stronger vitality if the scutellum stained.

Lakon's procedure was followed with the exception that the concentrations of the solution used were ½%, 1%, and 2% for two kinds of seed, and the length of time for presoaking and immersion in the tetrazolium salt solution was varied for some kinds of seed.

Seed samples used were tested in the Iowa State College Seed Laboratory. In all cases a germination test was made by approved methods to compare with the color test.

This paper reports the results obtained with the use of tetrazoliumchlo-

ride as a staining solution of the embryos of viable seeds in which germination processes have been initiated.

Results

Corn

For the initial studies with corn a sample with a known germinability of 98% under favorable conditions was selected. Seeds were soaked in water at room temperature from 5:00 P.M. to 8:00 A.M. the following day. Two hundred of the soaked seeds were then cut longitudinally through a median line perpendicular to the embryo side. Care was taken to split the plumule and radicle. One half of each kernel was saved and the entire set of 200 halves covered with a 1% solution of the tetrazoliumchloride. The Petri-dish container was immediately placed in a dark cabinet at room temperature. At the end of 15 minutes a faint pink coloration was noticeable in the embryo region of many seeds. At the end of 2 hours the entire number was examined and 99% showed bright carmine red, not only in the embryo including the plumule and radicle, but also in the scutellar region. The following day 400 more seeds of the same lot were soaked overnight and dipped in boiling water for 5 minutes. Two hundred were then sectioned, covered with the tetrazolium solution, and kept for 8 hours in the dark. No embryos changed color. The remaining 200 seeds were planted in sand, and none germinated. This preliminary test indicated that the tetrazolium solution was capable of differentiating at least between seeds that were nearly 100% germinable and those that were 100% non-viable. Several additional lots of seed corn produced in 1945 were tested with the same solution with results as shown (table I). At the same time a ½% solution was used on one lot as well as a 1% solution. There was no noticeable difference in the results obtained.

TABLE I

COMPARATIVE DETERMINATION OF SEED CORN VIABILITY BY APPROVED METHODS OF GERMINATION AND BY STAINING REACTION WITH 1% SOLUTION OF TETRAZOLIUMCHLORIDE

Sample no.	Staining		Viability in sand	
	No. of seeds	Coloration	No. of seeds	Germination
		%		%
5	200	90.0	200	90.0
22	200	90.0	200	89.5
23	200	86.5	200	90.5
39	200	22.5	200	23.5
40	200	9.0	200	7.0
1036	400	75.0	400	70.0
1072	400	86.2	400	82.0
1127	400	99.0	400	98.0
1169	400	85.0	400	81.0
1223	400	99.2	400	99.0
1228	400	97.5	400	90.0
1253	400	75.8	400	69.0
1298	400	90.8	400	90.0

SMALL GRAINS, SORGHUM, BUCKWHEAT, AND LEGUMES

Samples of barley, oats, wheat, sorgo, buckwheat, popcorn, peas, soybeans, and vetch were tested with tetrazolium solution in much the same manner as described for corn. The soaking period, both in water and in the solution, was varied. The seeds of small grain, sorgo, popcorn, and buckwheat were cut medianly through the long axis of the embryo. The seeds of pea, soybean, and vetch were cut between the cotyledons, bisecting the radicle longitudinally. The data (fig. 1) show that for 3 lots of barley, 2 lots of oats, wheat, buckwheat, soybean, and popcorn there was a close relationship between the percentage of stained embryos and of normal sprouts produced in the ordinary laboratory germination test in sand (table II).

The tests with sorgo indicated that the color reaction might not be entirely reliable for the seeds of this plant; however, more tests are necessary before proof can be established. Sorgo seed would probably respond in a manner similar to that of corn and cereals. A careful study of the degree of coloration of the embryo, as well as time of soaking in the solution, may result in a refinement of the method.

Seeds of legumes require different treatment than those of grasses. For example, a long period of soaking in the chloride solution results in a coating of reddish stain over the entire flat surface of each cotyledon, and the stain must be rubbed off before the radicle can be examined. Furthermore, there is no indication as to the condition of the epicotyl in peas and vetch seed or of the plumule in soybean seed, primarily because it is difficult to split the plumule or epicotyl longitudinally in the sectioning process. To determine by this method which bean seeds would produce "baldhead" seedlings is practically impossible. The primary root emerges early in the germination of legume seeds and before the epicotyl or plumule shows much growth. This condition may prevent any practical use of the staining method as a means of determining the germinability of such seeds. Further investigations are needed before conclusions can be drawn.

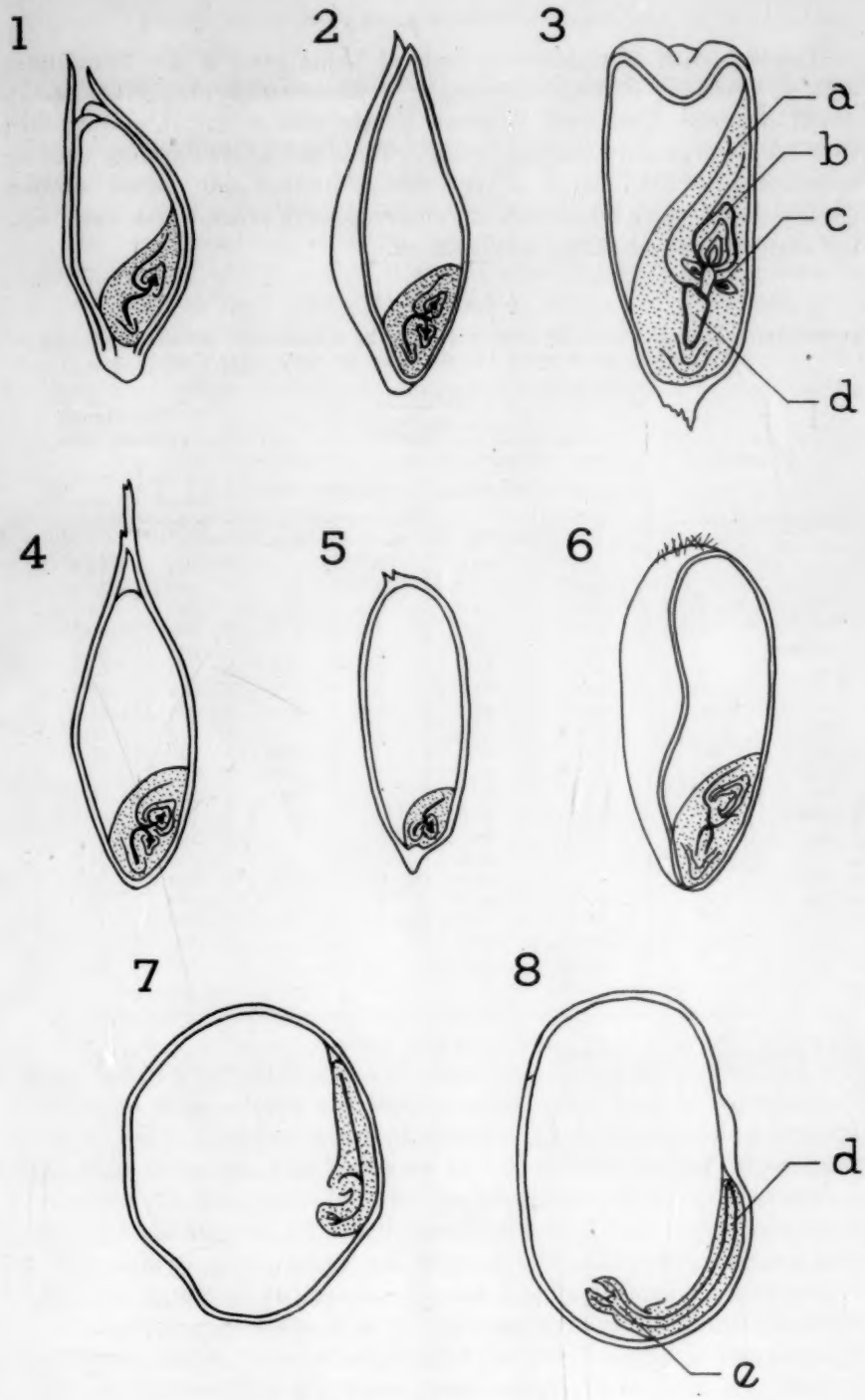
FIG. 1. Longitudinal sections of seeds showing area (heavy black or shaded portions) that stained carmine red when soaked in a ½% solution of 2,3,5-triphenyl-tetrazoliumchloride: *a*, procambial strand; *b*, coleoptile; *c*, adventitious root; *d*, primary root; *e*, plumule. Figure 1, 20×; figure 6, 12×; others, 6×.

1, 2, 4, 5, 6. Sections of Bahia grass, barley, oat, rice, and wheat seeds. Only those which showed the entire embryo and scutellum with carmine red stain were considered viable.

3. Corn kernel—seeds which stained carmine red in the scutellum and plumule regions down to and including the lateral root buds were considered viable. The stain was usually present throughout the scutellum and embryo.

7. Pea cotyledon—a section cut between the cotyledons and through the radicle. Those with a carmine red radicle were considered viable although no evaluation of the condition of the epicotyl could be made.

8. Soybean cotyledon—a section cut between the cotyledons and through the radicle. Those with a carmine red radicle were considered viable, although no evaluation of the condition of the plumule could be made.


1
2
3
a
b
c
d
4
5
6
7
8
d
e

Bahia grass

Determination of viability in seeds of Bahia grass is one of the most difficult problems in seed technology. Recommended procedures are to dehull the fresh fruit seed, to scrape lightly with a scalpel, and moisten with 0.1% potassium nitrate solution. These procedures are not entirely satisfactory as dehulling is a very tedious process and nitrate solution usually results in an abundance of fungous growth around each seed, making evaluation of sprouting very difficult.

TABLE II

Comparative determination of seed viability by laboratory germination and by staining reaction with 1% solution of tetrazoliumchloride

Sample		Staining test				Laboratory germination	
		No. seeds	Colored	Staining time		No. of seeds	Normal sprouts
			%	*hrs.*	*min.*		%
Barley	1A*	200	90.0	5		200	93.0
”	1A†	200	63.0	5		200	80.5
”	27C*	200	75.0	5		200	74.0
”	27C†	200	61.0	5		200	60.5
Buckwheat	1	200	98.5	4		200	95.0
Oats	36	179	83.2	2		200	94.5
”	38	200	75.0	2		200	89.5
”	Marion	200	94.0	1		400	98.0
”	Tama	200	47.0	1		400	50.0
Peas	55101	194	90.2		20	200	81.5
”	55105	192	91.6		20	200	78.5
”	55106	197	90.3		20	200	85.0
Popcorn	43610	200	32.0	2		200	27.5
Wheat	61	200	84.0	5		200	85.0
”	120	190	73.6	5		200	71.5
Soybean	1	200	100.0		30	200	96.0
Sorgo	9466	200	93.5	2		200	76.0 (80)‡
”	9467	200	75.5	2		200	49.0 (68)‡
”	49545	200	69.5	2		200	45.0 (51)‡
Vetch	1	200	58.0		15	200	75.5

* New crop.
† Old seed.
‡ Seed treated with Arasan.

Two types of tests using tetrazoliumchloride solution were undertaken. The first consisted of soaking the seeds from a pure seed fraction in water at 20° to 35° C. for 1 to 7 days. At the end of each day 3 lots of 50 seeds each were removed, sectioned, and soaked in ½%, 1%, and 2% solutions of tetrazoliumchloride. The results showed that a 2% solution was too strong, that there was little difference between the results obtained with a ½% or a 1% solution, and that after 3 days of soaking the percentage of colored embryos declined. The declining of the colored embryos suggests that seeds of Bahia grass, if soaked several days, begin to lose vitality. Such a response is not unexpected, because many seeds will not germinate in water.

The seeds absorb water and germination is initiated, but, if kept too long, decomposition occurs.

The second type of test with Bahia grass consisted of soaking the seeds 16 hours in distilled water at 20° C. The seeds were then placed in a Petri dish on moist filter paper and kept in a germinator with an alternating temperature of 20° C. for 16 hours and 35° C. for 8 hours. Immediately after the initial period of soaking and at subsequent intervals of 24 hours, 50 seeds were removed, sectioned, and placed in a 1% solution of tetrazoliumchloride. At the end of 8 and 24 hours the sections were examined and classified as light-stained, dark-stained, viable, and non-viable. Only those with the entire embryo and scutellum stained light or dark were considered viable (table III).

TABLE III

RESULTS OF SOAKING 50 CUT SEED SECTIONS OF BAHIA GRASS IN 1% SOLUTION OF TETRAZOLIUMCHLORIDE FOR 8 AND 24 HOURS*

No. of days after soaking	No. of dark-stained seeds	No. of light-stained seeds	Viable	Non-viable	Time in solution
			%	%	*hrs.*
0	2	48	100	0	8
	35	15	100	0	24
1	8	42	96	4	8
	37	13	96	4	24
2	29	20	96	4	8
	44	5	94	6	24
3	35	15	100	0	8
	47	3	98	2	24
4	22	24	88	12	8
	42	3	90	10	24
5	25	25	100	0	8
	40	10	100	0	24
6	31	19	96	4	8
	38	9	94	6	24

* Seeds were soaked 16 hrs. in water at 20° C., kept at temperatures alternating between 20° and 35° C. for period of 1 to 6 days, and sectioned.

In all cases the percentage of dark-stained embryos increased markedly as the period of immersion was increased from 8 to 24 hours. Since the embryos not deeply stained were colored throughout the scutellar region as well as in the embryo proper, they were considered viable. Among the lots kept in the germinator for 1 to 6 days and soaked 24 hours in the chloride solution, the differences in viability, on the basis of 50 seeds, were not significant. On the other hand, the large percentage of dark-stained seeds retained in the germinator for 2 to 4 days after soaking, indicated that such a procedure may be preferable. The viability by this method of this particular lot of Bahia grass seed appeared to be between 90% and 100%.

A germination test of this lot was made as follows: 100 seeds were dehulled, soaked 5 minutes in a 25% solution of chlorox, placed on moist filter papers in a Petri dish, and kept in a germinator with alternating tempera-

tures of 15° C. for 16 hours and 30° C. for 8 hours. At the end of 9 days all the seeds had produced strong, normal sprouts. Another sample of 200 seeds, treated in the same way except that the temperature alternated from 20° to 35° C., gave 95% germination in 5 days. The results indicated that the viability of this lot of seed was nearly 100% which agrees with the index of viability as measured by the tetrazoliumchloride method. They also suggested that alternating temperatures of either 15° to 30° C. or 20° to 35° C. were favorable for the germination of Bahia grass seed.

Rice seed

Two hundred seeds from each of four varieties of rice were soaked in water for 16 hours at room temperature. Each seed was sectioned longitudinally through the embryo, and one half of each placed in a 1% solution of tetrazoliumchloride for 4 hours. At the end of the soaking period the

TABLE IV

Comparative determination of the viability of rice seed by staining with 1% solution of tetrazoliumchloride and laboratory germination based on 200 seeds tested

Variety	Viability by staining	Laboratory germination		
		Between blotters	Between blotters 20°–30° C.	Sand
	%	%	%	%
Nira	66.0	65.5	55.0	70.0
Fortuna	87.0	84.0	77.5	81.0
Prelude	84.5	75.5	72.0	71.5
Zenith	94.5	90.0	78.0	86.5

sections were examined under a binocular microscope and all those with the embryo and scutellum stained light or dark red were classed as viable. Three lots of 200 seeds each from the 4 varieties were prepared and tested for germination by three methods: (1) between blotters, (2) presoaked 16 hours and planted between blotters, and (3) presoaked 16 hours and planted in sand. All tests for germination were made at 20° C. for 16 hours and 30° C. for 8 hours each day for 10 days (table IV). With the exception of the Prelude variety soaking before planting resulted in a higher percentage of germination, and germination in blotters or sand of presoaked seed agreed with the embryo color reaction.

Cotton seed

A few attempts were made to apply the tetrazolium color test to cotton seed. One difficulty was that some seeds after soaking overnight were hard and could not be sectioned. Such seeds presumably would not have absorbed water, and germination processes could not have been initiated. Many seeds that did absorb water when sectioned and soaked in a 1%

tetrazoliumchloride solution stained readily in the embryo region; but the seed coats separated easily from the embryos and cotyledons, and the latter further separated from each other. An evaluation of the color test, therefore, was difficult to make.

Discussion and summary

Determination of living embryos of seeds by means of the color indicator 2,3,5-triphenyl-tetrazoliumchloride as recommended by LAKON (**12**) was investigated, using seeds of several members of the grass family, three leguminous species, buckwheat, and cotton. A comparison of the percentage of stained embryos in a given lot with the percentage of normal sprouts obtained in a standard germination test showed close agreement in 13 samples of corn, two samples each of wheat and oats, three samples each of barley and rice, and one sample each of buckwheat, popcorn, soybean, peas, and Bahia grass. The comparisons for vetch, sorgo, two samples of oats and peas, and one sample of barley were not in close agreement, yet the differences in results shown by the two methods were not great.

With the exception of corn the criterion used for classifying grass seeds as viable was the almost complete staining of the embryo proper and scutellum. Absence of color in the radicle below the mesocotyl region or in the lower half of the scutellum, however, was not considered sufficient grounds for classification of a corn seed as non-viable.

In seeds of pea, soybean, and vetch it is impossible to section through the epicotyl or plumule region regularly. The only cut part, therefore, with which the solution can readily make contact, is the radicle. Development of a radicle in leguminous seeds without consideration of the plumule or epicotyl is insufficient evidence for classification as a normal seedling. For that reason the method investigated may not be applicable to seeds producing baldheads when they germinate.

The method appears to have some merit for seeds of Bahia grass which are difficult to germinate. It is recommended that the hulls be removed before placing the seeds to germinate. This is a tedious and time-consuming process. By the staining method it was possible to soak the seeds 16 hours, place them on a moist substratum at 20° to 35° C. for 3 or 4 days, cut them into sections, and place in the tetrazoliumchloride solution for 24 hours. More samples must be tested by this method before definite conclusions can be stated. When the hulling method is used it is desirable to soak the seeds in a 25% solution of chlorox to prevent the growth of surface molds.

A standard method of germination may not be the one that measures the true germinability of a particular kind of seed. For example, in the tests with four varieties of rice the standard method failed to measure the maximum germinability of the seed of three varieties. When the seeds were soaked and planted in sand or blotters, the percentage germination compared closely with the percentage of stained embryos that were soaked in the tetrazoliumchloride solution.

As pointed out in the introductory statement, tetrazoliumchloride is colorless but forms carmine red formazans upon reduction. The salt is an oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is presumably an indication of the presence of active respiratory processes in which hydrogen radicals are transferred to the tetrazoliumchloride. If further research should establish this hypothesis, the process should prove to be of general value in physiological experiments as well as an index of germinability of seeds. Because the measurements of "germinability" are indirect, however, and dependent upon all of the factors affecting the oxidation-reduction potential of the cells, careful standardization of procedure will probably be necessary.

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CHLOROPHYLL AND PROTEIN INTERRELATIONSHIPS IN *ANANAS COMOSUS* (L.) MERR.[1]

C. P. SIDERIS

(WITH FOUR FIGURES)

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Studies on a quantitative separation and analysis of chloroplastic matter by different investigators have revealed that chlorophyll is held by chemical forces on proteinaceous matter. This matter, named phyllochlorin by MESTRE (**17**), chloroplastin by STOLL (**37**), photosynthin by FRENCH (**8**), and chloroglobin by RABINOWITCH (**21**), is presumably identical with the substance composing the stroma of chloroplasts.

FREY-WYSSLING (**9**), discussing the composition of chloroplasts, stated that, according to GUILLERMOND, MANGENOT, and PLANTEFOL (**13**) and SHARP (**24**), the structure of the chloroplasts of higher plants is homogeneous and not granular with small green particles imbedded in a colorless stroma, as postulated by MEYER (**18**) and SCHIMPER (**22**), and that the granulations are artifacts. According to HEITZ (**15**), the grana are not spheres but disks oriented parallel to the surface of the disk-shaped particles which alone contain the chlorophyll. NOACK (**20**) observed that colloidal chlorophyll does not show fluorescence except when adsorbed in monomolecular layers. Also, EULER *et al.* (**7**) have reached a similar conclusion from determinations of the quantity of chlorophyll in one single plastid.

Methods

The isolation of chloroplasts or of chloroplastic matter has been effected in various ways by different investigators (**3, 5, 8, 11, 12, 14, 17, 19**) and with somewhat similar results.

ISOLATION OF CHLOLOPLASTIC MATTER

In former studies (**25**) the leaves of *A. comosus* were segregated into groups represented by old (B), mature (C), active (D), and young (E) leaves. In *A. comosus*, as in all monocotyledonous plants, the old (B) leaves occupy positions at the basal end of the stem, the young (E) at the apical end, and the mature (C) and active (D) at the medial section. Such leaves are cut for many studies into four or five cross sections in order to segregate tissues of different stages of development and physiological function. The basal sections (no. 1) of such leaves are lacking in chlorophyll, and in leaves which have not completed growth they are composed mostly of meristematic tissues. The chlorophyllous region of the blade is divided into terminal (no. 5), medial (no. 4) and basal (no. 3) sections and the neck

[1] Published with the approval of the Director as Technical Paper no. 169 of the Pineapple Research Institute, University of Hawaii.

or region between the non-chlorophyllous (no. 1) and chlorophyllous blade (nos. 3, 4, 5) is known as the transitional section (no. 2).

In this study the isolation of chloroplastic matter was made as follows: One hundred grams of tissues from the chlorophyllous section (no. 4) of freshly collected active (D) leaves of *A. comosus* var. smooth Cayenne (25) cut approximately 2 mm. wide were placed with 250 ml. of 0.06 N NaOH in a Waring mixer and macerated at high speed for ten minutes. The mixture was squeezed by hand pressure through 200-mesh silk cloth or Canton flannel. The residue in the cloth was wetted with 25 ml. of 0.06 N NaOH and squeezed at successive intervals until all green color was removed from the residue.

The residue was discarded and the extract placed in 100 ml. "oil" centrifuge tubes and centrifuged for the first 5 minutes at about 750 r.p.m., the next 2 minutes at about 1100 r.p.m., and the last 3 minutes at about 1500 r.p.m. The gradual increases of centrifugal speed caused starch to settle at the bottom of the container with the least admixture of chloroplastic matter, while the latter remained in the supernatant liquid.

The supernatant liquid, containing chloroplastic matter, was transferred to clean centrifuge tubes without disturbing the precipitated starch, treated with acetic acid to pH 4.0 (1 ml. or more of 6 N glacial acetic acid), agitated gently to effect mixing, and then centrifuged at different speeds as mentioned. The supernatant liquid was decanted and the precipitate examined carefully for starch granules in the bottom layer of the container. When starch was detected, the precipitate was treated with 75 ml. 0.06 N NaOH, agitated to effect uniform suspension of the precipitate, and centrifuged as described. The supernatant liquid containing chloroplastic matter was removed and the precipitated starch discarded. The chloroplastic matter was flocculated with acetic acid as before and centrifuged.

To facilitate transferring the chloroplastic matter from the tubes to volumetric flasks, 1 ml. of 0.06 N NaOH was added, the contents agitated and poured into the flask. The alkaline suspension was acidified with acetic acid to pH 4.5, the flask filled to the mark with H_2O and placed in the refrigerator. The concentration of the contaminant sodium acetate could be reduced by removal of the clear supernatant liquid at daily intervals and replacement with H_2O. The mixture was agitated before withdrawing samples for analysis.

Chlorophyll was extracted from the chloroplastic matter with acetone and determined in a Klett-Summerson photoelectric colorimeter using a light filter no. 60. The proteinaceous residue was analysed for total nitrogen.

Results

Analyses of chloroplastic matter

Analyses of the chloroplastic matter from the leaves of *A. comosus* yielded 6.5 mg. of chlorophyll and 22.3 mg. of nitrogen per 20 grams of fresh tissue, the latter being equivalent to 140.0 mg. of protein (22.3×6.25).

HANSON (**14**) claimed that one tetrad of chlorophyll molecules (M.W. $900 \times 4 = 3600$) is associated in the cell with one molecule of chloroplastic globulin (M.W. 68,000). Thus Hanson's estimated ratio of chlorophyll to protein is equal to 0.0530, *i.e.*, $3600 \div 68{,}000$, whereas that obtainable in this study was 0.0465, *i.e.*, $6.5 \div 140.0$, the latter being 14.0% lower than the theoretical. The difference between Hanson's theoretical ratio and that obtained in our study may be due in part to incomplete extraction of chlorophyll—the extract was estimated to contain approximately 95% of the total chlorophyll in the chloroplastic matter because the latter was faint greenish yellow after repeated extractions.

ANALYSES OF TISSUES WITHOUT EXTRACTION OF PROTOPLASMIC MATTER

Chlorophyll/protein ratios from other studies (**26, 27**) show gradients of ascending values from the basal to the terminal sections of leaves (table I). It will be noted that the ratios of chlorophyll to protein for section no. 4 of the active (D) leaves (**25**) were greater in the studies referred to above, where no separation of the chloroplastic matter from the tissues was effected but protein-N was estimated as the difference between total-N and soluble-N, than the value 0.0465 in the present study or the theoretical value 0.053. The results in table I indicate that the amounts of protein in relation to chlorophyll were about one-half as great as in the isolated chloroplastic matter and suggest a low rate of protein synthesis, a high rate of protein breakdown, or a low rate of chlorophyll reduction.

In sections no. 2 and no. 3, which are chronologically and physiologically less advanced than no. 4 and no. 5, the protein content of the tissues in relation to chlorophyll was less, either because of shorter periods of deposition and differences in the physiological functions of the tissues or of greater amounts of extracted protein, due to high pH values in sections no. 2 and no. 3 than in no. 4 and no. 5, which reduced the degree of association of protein with chlorophyll (**23**).

In general protein correlated with the chlorophyll content of tissues (figs. 1, 2, 3, and 4). The coefficient of correlation, calculated according to SNEDECOR (**34**) from $r = Sxy/\sqrt{(Sx^2)\ (Sy^2)}$ was statistically significant under experimental conditions which were favorable to plant growth. Chlorophyll/protein ratios in many chlorophyllous sections were in agreement with the theoretical ratio but in others, mostly partly chlorophyllous, they were either higher or lower, presumably because of the methodological shortcomings stated above.

From previous publications (**27, 28**), depicting relationships between chlorophyll and protein in cultures supplied with equal amounts of nitrogen as NO_3 or NH_4 but with different amounts of iron, correlation between chlorophyll and protein in all cultures is shown (fig. 1). The coefficient of correlation for the minus-iron cultures in the ammonium series was better than for the minus-iron cultures in the nitrate series, because the intake of iron, which occurred as impurity in the C.P. salts of the nutrient solu-

TABLE I

PROTEIN-N, CALCULATED PROTEIN (PROTEIN-N × 6.25) AND CHLOROPHYLL OF FRESH TISSUE; ALSO, CHLOROPHYLL/PROTEIN RATIOS IN DIFFERENT LEAF SECTIONS OF ONE-YEAR-OLD *Ananas comosus*

Leaf sections		Nitrate series*				Ammonium series†			
		Prot.-N	Protein	Chlorophyll	Chlor./Prot.	Prot.-N	Protein	Chlorophyll	Chlor./Prot.
		mg./gm.	*mg./gm.*	*mg./gm.*		*mg./gm.*	*mg./gm.*	*mg./gm.*	
Mature (C)	1	0.12	0.75	.....	.....	0.38	2.38	.....	.....
	2	0.37	2.31	0.35	0.151	0.86	5.37	0.50	0.093
	3	0.80	5.00	0.59	0.118	1.66	10.38	0.83	0.080
	4	1.00	6.25	0.69	0.110	2.14	13.38	1.05	0.078
	5	1.20	7.50	0.86	0.114	2.50	15.63	1.22	0.078
Active (D)	1	0.19	1.19	.....	.....	0.34	2.12	.....	.....
	2	0.31	1.94	0.28	0.144	0.61	3.81	0.30	0.079
	3	0.62	3.88	0.46	0.119	1.45	9.05	0.56	0.062
	4	1.22	7.62	0.60	0.079	2.10	13.13	0.78	0.059
	5	1.58	9.87	0.74	0.075	2.48	15.50	1.02	0.066
Young (E)	1	0.39	2.44	.....	.....	0.33	2.06	.....	.....
	2	0.39	2.44	0.12	0.049	0.64	4.00	0.20	0.050
	3	0.66	4.12	0.33	0.080	1.19	7.44	0.40	0.054
	4	1.25	7.82	0.50	0.064	1.92	12.00	0.78	0.065

* Grown in solution cultures supplied with nitrate.
† Grown in solution cultures supplied with ammonium salts.

tions, was greater from the ammonium-N series with ultimate lower pH values than from the nitrate-N series with higher ultimate pH values than at the beginning of the absorption period.

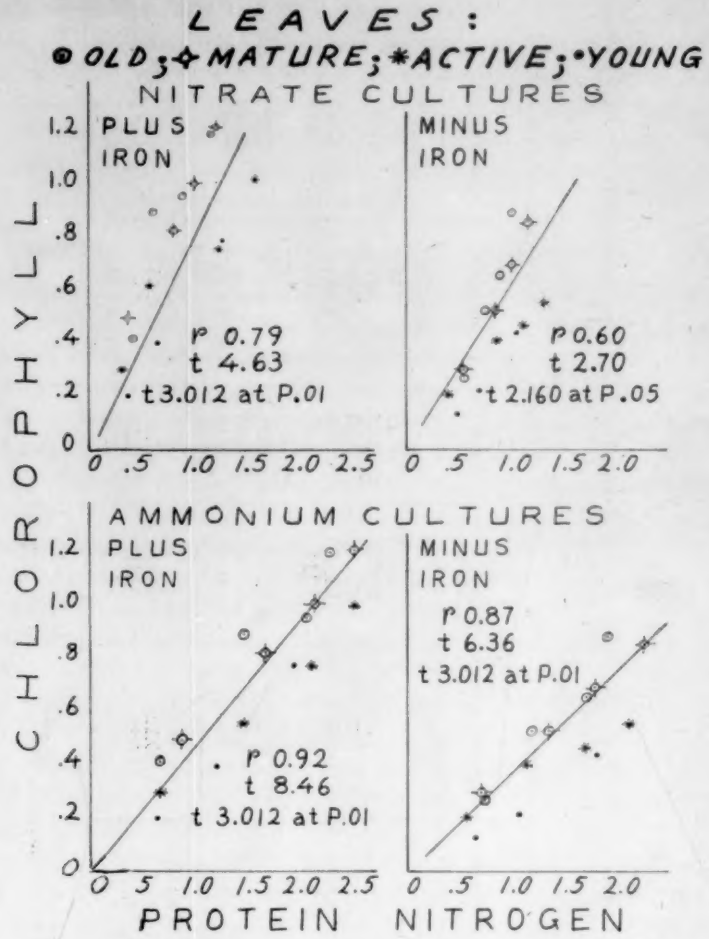

FIG. 1. Correlation coefficient, *r*, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. comosus* grown in solution cultures supplied with or without iron and with equal amounts of nitrogen either as NO_3^- or NH_4^+.

From previous publications (**31, 32**), pertaining to the effects of 140.0 *vs.* 2.8 mg. of nitrate or ammonium nitrogen in nutrient solutions on the chlorophyll and protein content of different leaf sections, highly significant correlation coefficient values are shown (fig. 2). In this figure as in figures 1, 3, and 4, each point represents the mean value of similar sections in the different leaf groups instead of individual sections. It will be noted that protein synthesis for the low-N cultures was greatly inhibited by nitrogen

deficiency, which also caused a parallel reduction in the chlorophyll content of the tissues (fig. 2).

From previous publications (**28, 29**), depicting the effects of 205 *vs.* 4 mg. of potassium per liter of nutrient solution on the chlorophyll and protein content of different leaf sections, it is shown that correlation coefficient values were highly significant for all cultures and may suggest that potassium played no direct role in protein or chlorophyll synthesis (fig. 3).

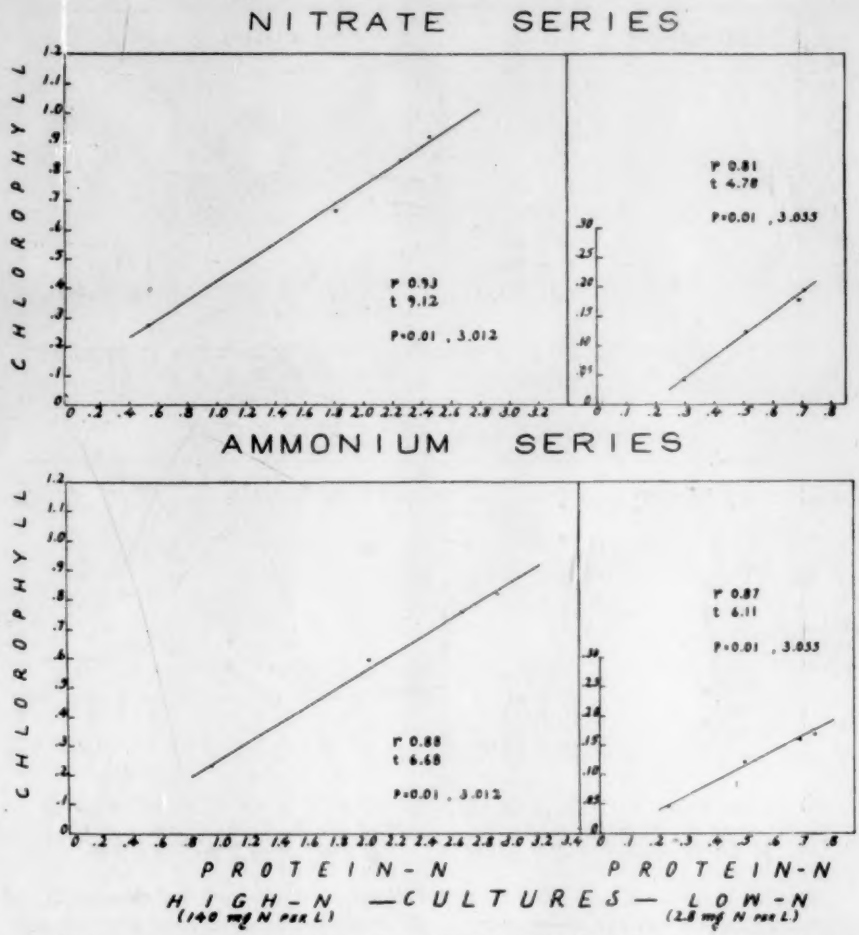

FIG. 2. Correlation coefficient, *r*, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages (the values are means for similar sections of leaves of different ages) of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter, supplied either as NO_3^- or NH_4^+.

From a previous publication (**25**), depicting chlorophyll and protein relationships in two shoots from the same plant, of which one was exposed to seasonal light and the other was covered by a bag of black cloth for 56 days, statistically significant correlation coefficient values for the exposed

plant at $P = 0.05$ level but not at $P = 0.01$ level are indicated (fig. 4). Correlation between chlorophyll and protein was lacking for the covered plant, presumably because darkness inhibited chlorophyll formation. It may be noted, however, that some sort of relationship existed between chlorophyll and protein in the old (B) and in the mature (C) leaves which at the time

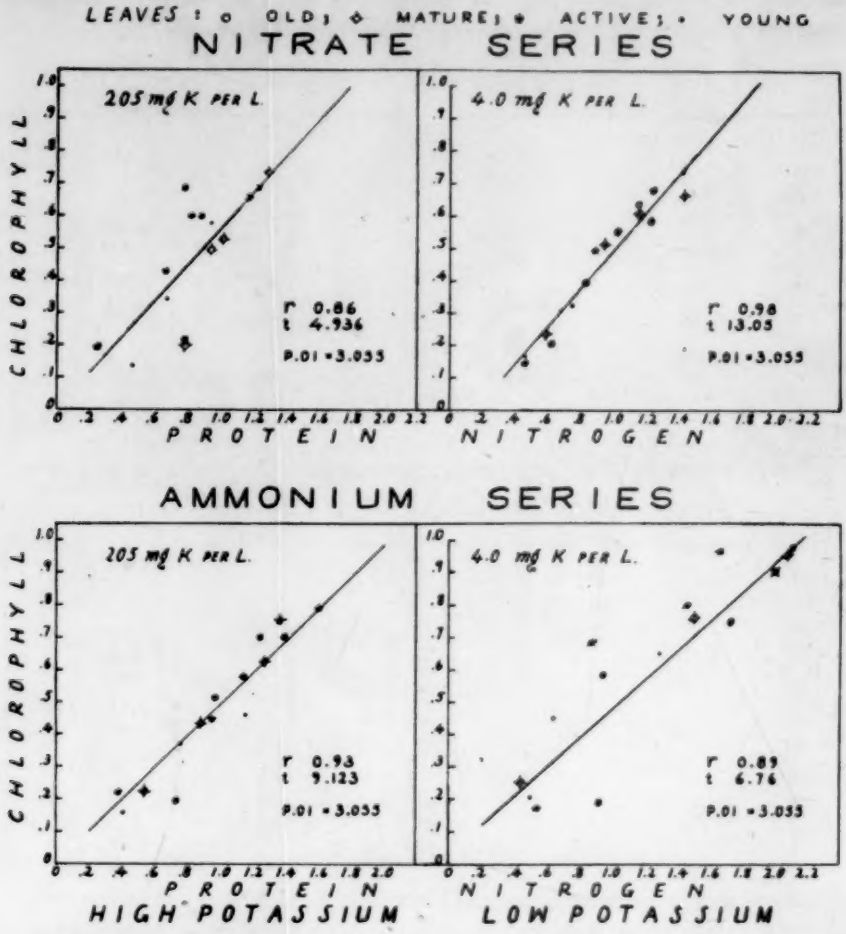

FIG. 3. Correlation coefficient, r, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. comosus* grown in solution cultures with 200 or 4 mg. of K per liter and supplied with equal amounts of nitrogen as NO_3^- or NH_4^+.

of covering had approximately completed growth, but not in the active (D) and young (E) leaves which developed in darkness, lacked chlorophyll, and contained relatively little protein-N.

The findings in table II suggest that in cultures of *A. comosus* supplied with nitrate as the source of inorganic nitrogen, protein deposition in the

TABLE II

PROTEIN-N, CALCULATED PROTEIN (PROTEIN-N × 6.25) AND CHLOROPHYLL OF FRESH TISSUE; ALSO, CHLOROPHYLL/PROTEIN RATIOS IN DIFFERENT LEAF SECTIONS OF ONE-YEAR-OLD *Ananas comosus* SISTER SHOOTS

LEAF SECTIONS		EXPOSED*				COVERED†			
		PROT.-N	PROTEIN	CHLOROPHYLL	CHLOR./PROT.	PROT.-N	PROTEIN	CHLOROPHYLL	CHLOR./PROT.
		mg./gm.	*mg./gm.*	*mg./gm.*		*mg./gm.*	*mg./gm.*	*mg./gm.*	
Mature (C)	1	0.368	2.30	.....	.....	0.245	1.53	.....	.....
	2	0.502	3.14	0.071	0.0226	0.350	2.19	0.017	0.0078
	3	0.646	4.03	0.220	0.0546	0.394	2.46	0.078	0.0318
	4	0.870	5.44	0.320	0.0589	0.516	3.23	0.075	0.0232
	5	0.870	5.44	0.272	0.0500	0.525	3.28	0.102	0.0310
Active (D)	1	0.313	1.95	0.000	.....	0.327	2.04	0.000	.....
	2	0.340	2.12	0.035	0.0165	0.310	1.94	0.000	.....
	3	0.438	2.74	0.126	0.0460	0.348	2.17	0.000	.....
	4	0.682	4.26	0.128	0.0300	0.464	2.90	0.007	0.0024
	5	0.885	5.54	0.294	0.0530	0.464	2.90	0.082	0.0280
Young (E)	1	0.400	2.50	0.000	.....	.....	.....	0.000	.....
	2	0.317	1.98	0.038	0.0192	.....	.....	0.000	.....
	3	0.514	3.22	0.048	0.0149	0.347	2.16	0.000	.....
	4	.....	.....	.....	.....	0.206	1.29	0.020	0.0155

* Exposed to seasonal sunlight.
† Covered with a black cloth for 56 days.

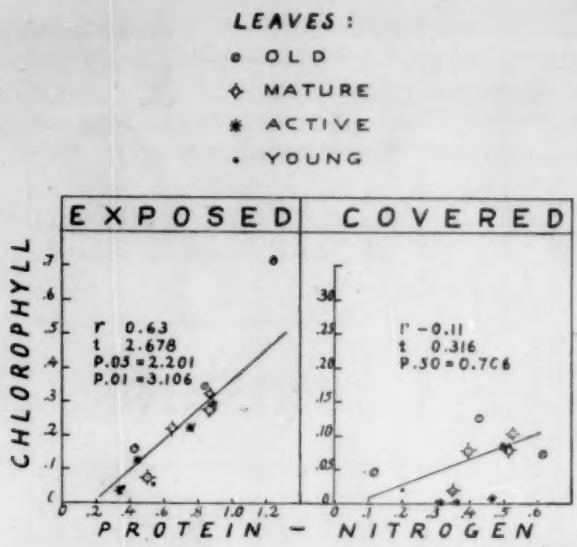

FIG. 4. Correlation coefficient, *r*, and its statistical significance for cholorophyll and protein in different sections of the leaves of different ages of *A. comosus* sister shoots grown on the mother plant, of which one was kept in darkness for 56 days by covering with a black cloth; and the other was exposed to seasonal light.

chlorophyllous sections (nos. 3, 4, and 5) was in much greater amounts than in the basal non-chlorophyllous sections (no. 1). This may indicate that protein deposition was simultanteous with chlorophyll production, presumably because of nitrate reduction and assimilation and carbohydrate synthesis in these sections.

In variegated leaves of *Ananas bracteatus* less protein-N was present in the non-chlorophyllous than chlorophyllous areas and the ratios of chlorophyll to protein were generally lower in the former than latter areas (table III). Chlorophyll to protein ratios in the green areas of leaves no. 2 and

TABLE III

CHLOROPHYLL AND PROTEIN-N OF FRESH TISSUE AND CHLOROPHYLL TO PROTEIN (PROTEIN-N × 6.25) RATIOS IN THE NON-CHLOROPHYLLOUS OR CHLOROPHYLLOUS AREAS OF VARIEGATED LEAVES OF *Ananas bracteatus* GROWN UNDER FIELD CONDITIONS

LEAVES		CHLOROPHYLL	PROTEIN-N	PROTEIN	CHLOR. / PROT.
No.	COLOR				
		mg./gm.	*mg./gm.*	*mg./gm.*	
1	White	0.068	0.28	1.75	0.039
1	Green	0.301	1.11	6.93	0.043
2	White	0.086	1.34	9.37	0.010
2	Green	0.729	2.13	13.30	0.055
3	White	0.083	0.81	5.06	0.016
3	White and green	0.231	1.07	6.67	0.035
3	Green	0.351	1.03	6.43	0.055

no. 3 had attained the theoretical value of 0.055, but in leaf no. 1 the observed ratio was about 22% lower than the theoretical value.

Discussion

STILES (**36**), SPOEHR (**35**), RABINOWITCH (**21**), WEIER (**38**), BURKHOLDER (**4**), GAFFRON (**10**), and FREY-WYSSLING (**9**) have presented the various aspects of photosynthesis insofar as it concerns chlorophyll and chloroplastic proteins either as a coordinating system or as independent systems.

Sugar synthesis in green plants by photosynthetic activity is effected by reduction of carbon dioxide with evolution of oxygen. Carbon dioxide reduction takes place in other organisms lacking chlorophyll which may or may not be sensitive to light, according to GAFFRON (**10**) and WERKMAN and WOOD (**39**), suggesting that the proteinaceous matter in the cell (which may function similarly to the proteinaceous stroma in the chloroplasts), and not the chlorophyll is the locus for carbon dioxide reduction. Experimental evidence (**6**) on the time required for the photosynthetic mechanism corroborates the view that carbon dioxide reduction may be effected in the absence of chlorophyll proper in some other adjacent region of the system and possibly in the chloroplastic stroma. HÖBER (**16**), in an attempt to explain certain observations on studies in photosynthesis, states his point of view as follows: "The time element, possibly associated with long range forces, may be necessary to explain the observed fractional quantum efficiency for a large assembly of chlorophyll molecules in the interior of the chloroplast to absorb four light quanta simultaneously and then to transmit the electronic excitation energy from one molecule to the next by some sort of resonance effect, until it reaches a point on the surface occupied by an adsorbed CO_2 molecule."

The data on protein-N and chlorophyll content in various leaf sections of *A. comosus*, in previous publications (**27, 28, 29, 30, 31, 32**), indicate in general gradients which increased from the basal chlorophyll-free semimeristematic no. 1 section to the terminal more or less senescent no. 5 sections. In plants supplied with insufficient amounts of iron, nitrogen, or light the chlorophyllous sections of the leaves contained relatively equal or slightly greater amounts of protein-N than the non-chlorophyllous basal no. 1 sections. Inhibition of chlorophyll synthesis by deficiencies in iron, light, or nitrogen caused a parallel reduction of protein depositions in the chlorophyllous tissues.

In plants adequately supplied with iron, light, or nitrogen, chlorophyll to protein ratios were approaching the theoretical value, although they fluctuated considerably for different sections of the leaves. Lowest ratios and ratios nearer the theoretical value were obtained mostly in the distal more mature regions of the leaves, *e.g.*, sections 4 and 5, but in the proximal sections 2 or 3 which are less mature, the ratios were ordinarily higher. It is probable, therefore, that protein depositions in the latter were either

limited by unknown physiological causes or that some protein might have been incorporated in the soluble organic-N fraction and the reported protein values were not sufficiently high to yield the theoretical ratio, 0.055. It was observed in subsequent studies, which will be reported elsewhere, that from 12.0% to 28.0% of the total water-extractable nitrogen is proteinaceous and may be precipitated by acetic acid, while from 1.0% to 12.0% is proteose-N which is precipitable by trichloroacetic acid. In many analyses reported above, all proteose-N was incorporated in the soluble organic-N fraction. The subtraction of the latter from total-N to yield protein-N by difference lowered the actual proteinaceous values from 2.0% to 25.0% on account of the inclusion of proteose-N in the soluble organic-N fractions. If this correction be taken into consideration, many of observed chlorophyll/protein ratios approach the theoretical value.

Studies of protein structure by ASTBURY (**1, 2**) indicate that the fibrous proteins, have as the unit of structure a polypeptide grid whose normal equilibrium configuration is not flat, but buckled, that is, the main chains are thrown into a series of intramolecular folds lying in planes transverse to the cross-linkage of the grid, and its basis of the long-range elasticity is the capacity to unfold and refold. Moreover, additional evidence pointing to a simple system of close packing of the side chains, the polars on one side of the main chain and the non-polars on the other, suggests that such close packing results in the formation of protein laminae which may appear alone or sandwiched with accessory groupings, such as lipoid laminae. SCHULMAN (**23**) has shown that, in lipoproteins, the pH of the solution with values above or below the isoelectric point of the protein may increase or decrease the the relative degree of association or combination of lipoids with proteins.

In *A. comosus* the pH and titrable acidity values of the sap from different leaf sections vary considerably (**26, 27, 29, 31**). Gradients of titrable acidity and pH of the various leaf sections, influenced by diurnal changes and rates of respiratory activity (**33**), increased in the former but decreased in the latter from the basal to the terminal sections during the hours of darkness or low light, while at times of full sunlight, when photosynthetic activity was at maximum, they either disappeared or became slightly reversed. Thus the different conditions provided in the cell by the constantly changing acidity or pH may increase or decrease the degree of association between chloroplastic proteins and chlorophyll. Hence, the divergence between observed and theoretical chlorophyll/protein ratios may be explained by the effects of pH on the chlorophyll combining power of the chloroplastic proteins of different leaf sections.

Summary

There was positive correlation between chlorophyll and protein in the chlorophyllous regions of plants supplied with adequate amounts of nutrients and seasonal light.

Insufficiency of iron, light, and nitrogen decreased considerably the amounts of chlorophyll and protein in the chlorophyllous regions of leaves.

Potassium in the nutrient solutions supplied in small or high amounts had no measurable influence on the relations of chlorophyll to protein.

Ratios of chlorophyll to protein in the distal relatively mature leaf sections, where both substances reached maximal content, approached the theoretical value of 0.055 of Hanson, but in the proximal less mature sections chlorophyll/protein ratios indicated greater amounts of chlorophyll in proportion to protein.

Protein-N/soluble organic-N ratios were approximately 1:1 in the basal (no. 1) non-chlorophyllous sections and 3:1 in the chlorophyllous (no. 4) sections, indicating that protein-N rather than soluble organic-N is directly related to chlorophyll.

These findings suggest that chlorosis, excluding variegation, in plants is a state of incompatible chlorophyll and protein relations in the chloroplast due to deficiencies of substances essential for the synthesis of chlorophyll or proteins, or to conditions (high Mn) inhibitory for the synthesis of such substances or for the proper operation of the chlorophyll-protein system. This state may be restored to normalcy either by the elimination of the deficiencies or the adverse conditions.

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RADIOACTIVE PHOSPHORUS PROCEDURES AS APPLIED TO SOIL AND PLANT RESEARCH[1]

C. L. COMAR AND J. R. NELLER

(WITH ONE FIGURE)

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The importance of phosphorus in soil–plant relationships is well recognized, as evidenced by the magnitude of the phosphate fertilizer industry in the United States. Knowledge is incomplete, however, regarding the behavior of phosphorus in the soil, its availability for plant uptake, and the extent to which it is affected by the numerous ever-present variables. The isotopic tracer technique, in addition to sensitivity, simplicity, and rapidity, possesses a fundamental advantage over the standard chemical methods in that phosphorus added to a system can be distinguished from that already present.

In 1940 BALLARD and DEAN (**3**), who reported on the use of radioactive phosphorus in soil studies, used an electroscope for detection purposes and made measurements on soil, plant material ash, and on intact plants. In 1941 BALLARD and DEAN (**4**) described the application of these procedures in biological measurements of the retention of applied phosphorus by soils; they obtained close agreement between phosphorus fixation when determined by the standard water equilibrium system method, and when determined by the absorption by 19-day-old tomato plants of radioactive phosphorus. They were unable to obtain correlation between the standard method and the use of radioactive phosphorus in the chemical estimation of phosphorus fixation. No data as to the sensitivity of the detection procedures used or the specific activities of the original solutions were presented. The use of radioactive phosphorus in short-term plant physiological studies has been reported by several workers (**1, 5, 7, 8**).

This technique has not yet been extensively used, principally because of an inadequate source of radioactive materials and the lack of detection equipment during the war years. With cyclotron output now available for peacetime research, however, and with the reasonable expectation that atomic energy plants will soon be used for the production of radioactive isotopes, the main obstacle will be removed (see Science, June 14, 1946). Experience has indicated that field studies are economically feasible in addition to the laboratory and greenhouse scale of experimentation.

It is the purpose of this paper to describe advantageous procedures for the use of radioactive phosphorus in soil and plant studies and to present results with a series of Florida soils. Data are given on the sensitivity of the method, since this factor in consideration with an estimate of the expected

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uptake will determine the practical maximum time limit of the experiment and the activities which must be used.

Materials and method

Radioactive material was supplied by the Massachusetts Institute of Technology, Radioactivity Center, and was prepared by bombardment of phosphorus with deuterons. It was obtained as a purified solution containing 4.2 mg. phosphorus in the form of H_3PO_4, with a half-life of 14.3 days and an initial specific activity of 0.95 millicuries per mg. Using the 15-ml. cell described below, the measurements were sensitive to 0.00015 micrograms phosphorus at 12 days after preparation.

The activity was measured with a Geiger-Mueller counter apparatus (No. 3 combination purchased from Herbach and Rademan, Inc., Philadelphia, Pa.). A dipping type counter tube was employed constructed similarly to that described by BALE, HAVEN, and LEFEVRE (**2**) (purchased from Distillation Products, Inc., Rochester, N. Y.). BALE *et al.* described a special mount for holding the counter tube; they used a rack and pinion device for supporting and adjusting the glass cell which contained the solution to be measured. Satisfactory results were obtained, however, by holding the counter tube with an ordinary clamp and ring-stand and by supporting the cell with a pivoted beaker shelf which could be fixed at the desired height and swung aside for removal of the cell (fig. 1). The sensitive portion of the counter tube is a cylinder about 51 mm. in length and 11 mm. in diameter. For maximum sensitivity, a test tube of 15 mm. inside diameter and about 60 mm. in length should be used with 2 ml. of the unknown solution. However, when the samples contain sufficient activity it is more convenient to use larger cells; in the work reported here 15 ml. of solution were used in cells of 22.4 mm. inside diameter and 63 mm. length. The sensitivity was decreased about fourfold by using the 15-ml. cells instead of the 2-ml. cells. For the actual measurement the cell was supported in paraffin contained in a 400-ml. beaker; the cell was held firmly, but it was removable for interchange of samples. No significant differences were observed when the cell was centered with the counter tube merely by inspection or when different cells of the same dimensions were used. The same paraffin-containing beaker should be used for any given series of measurements, since it is difficult to get any two beakers to support the cells at exactly the same height. With the counter tube and beaker shelf fixed in position, and using a given volume of solution in cells of identical dimensions in the same paraffin-containing beaker, reproducible results were obtained.

The background, due primarily to cosmic radiation, averaged 12 counts per minute for the set-up as described and was usually measured twice during the day. Each solution was measured for 10 minutes or longer, depending on the activity contained, and net counts of 4 per minute or less were not considered significant.

The absolute radioactive phosphorus content was obtained by the use of

standard solutions, prepared by using known amounts of the original radioactive phosphorus solution, carried through the regular procedure for the given experiment. The calibration curve was then constructed by plotting the weight of phosphorus against the count per minute. Instead of correcting for decay by calculation back to some zero time, a calibration curve was run during the middle of each working day for use with the samples measured on that day. Actually about 3 points were sufficient to determine the curve for routine work, and the position of the curve from day to day indicated that the instrument was operating properly.

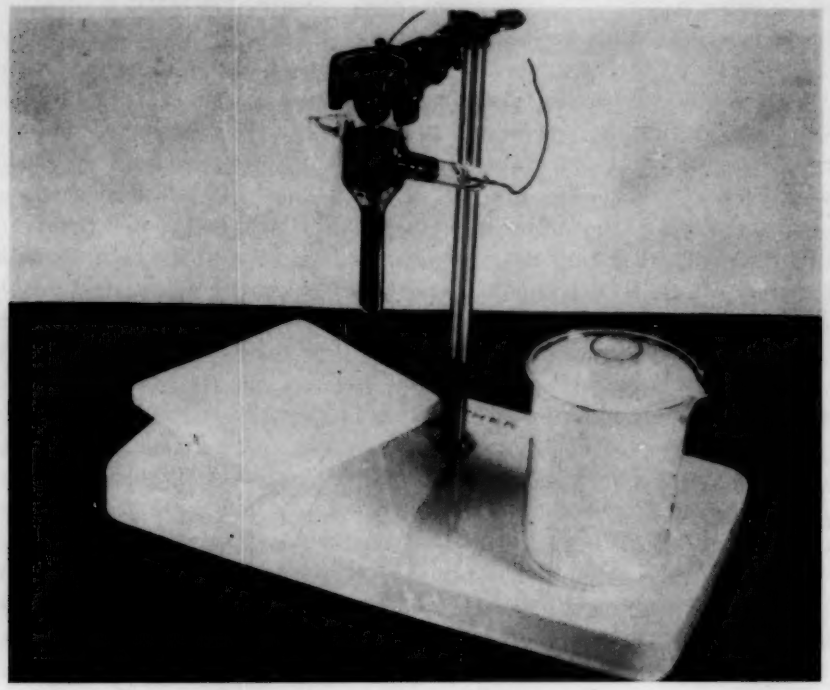

FIG. 1. Apparatus for measurement of radioactivity in solutions. Geiger-Mueller counter tube, swinging beaker shelf, and 15-ml. cell supported in 400-ml. paraffin-containing beaker.

The amount of radioactive material needed for a given experiment is fixed by (a) the sensitivity of the detection procedure at the time the measurements are to be made and (b) the amount of the isotope which will have accumulated in the sample. If the latter can be estimated, then the appropriate dosage can be calculated; the following example will illustrate how this was done for some of the experiments. In the greenhouse and lysimeter-jar studies it was expected that even those plants in soils of high fixing capacity would take up not less than 1% of the added phosphorus. It was decided that the measurements would be made not more than 3 months after the date on which the sensitivity had been determined as 0.00015 micrograms phosphorus; due to radioactive decay the sensitivity after 3 months would be

about 0.01 micrograms. For optimum measurement the sample should contain at least 10 times the minimum activity, and, since 2 samples were to be taken from each jar, it was calculated that an aliquot of the original solution containing about 20 micrograms of phosphorus added to each jar would be in the proper range.

Results and discussion

Phosphorus fixation by soils

The degree of phosphorus fixation by soils may be determined satisfactorily by chemical methods of analysis. The advantages in the use of the radioactive-tracer method are (a) simplicity and rapidity, (b) use of minute amounts of added phosphorus and (c) elimination of need to correct for behavior of original phosphorus in the soil.

The general procedure was as follows: an extracting buffer solution of dilute acetic acid and sodium acetate as described by Morgan (**6**) was employed. For the equilibrium studies appropriate amounts of radioactive phosphorus in the form of Na_2HPO_4 plus the inert phosphorus as KH_2PO_4 were incorporated in the extracting solution, and 50 ml. of this solution were agitated with 10 gm. of soil in a mechanical shaker for 30 minutes. The mixture was filtered, and the amount of radioactive phosphorus recovered was determined by direct measurement on the filtrate. Some of these solutions were analyzed chemically, and corresponding values for recovery were calculated by correcting for the phosphorus appearing in the filtrate when none was added originally.

In the extraction experiments 10 ml. of solution, containing the active and inert phosphorus, were added to the soil sample, which was then air dried, agitated with 50 ml. extracting solution, and carried through the procedure as described above.

Table I shows the phosphorus recoveries from the various soil types as obtained by the use of the different procedures; the values are the averages of duplicate determinations in all cases. The values from chemical analysis were in fair agreement with those obtained by the isotope technique, and the rating of the soils as to phosphorus fixation was the same by either method. Where the soils were air-dried after the addition of the phosphorus, the recovery was decreased in all cases, especially with the sandy loams and clay types. In general, there was a greater percentage of phosphorus recovered at the 200-p.p.m. level than at 50 p.p.m., particularly with fine sands; however, there was little difference with the Dunbar very fine sandy loam and Coxville clay. At the 400-p.p.m. level the phosphorus recovery was generally greater than at 200 p.p.m. although there appeared to be no simple proportionality between the initial amount added and the percentage recovery.

Phosphorus uptake by the plant

The value of an extraction procedure is determined by the extent to which the extracting solution removes those forms of phosphorus which are of

TABLE I

RECOVERY OF PHOSPHORUS ADDED TO THE SURFACE HORIZONS OF VARIOUS TYPES OF SOIL, USING ACETIC ACID–SODIUM ACETATE EXTRACTING SOLUTION

SOIL TYPE	AMOUNT PHOSPHORUS ADDED	PERCENTAGE RECOVERY OF PHOSPHORUS		
		CHEMICAL ANALYSIS OF EQUILIBRIUM SOLUTION	RADIOACTIVE PHOSPHORUS IN EQUILIBRIUM SOLUTION	RADIOACTIVE PHOSPHORUS IN EXTRACTION AIR-DRY SOIL
	p.p.m.	%	%	%
Leon fine sand	50	70	76	52
	200		88	61
	400		83	66
Hernando fine sand	50	54	51	16
	200		68	23
	400		76	25
Portsmouth fine sand	50	39	45	16
	200		59	25
	400		66	31
Norfolk loamy sand	50	43	43	6
	200		61	10
	400		66	11
Dunbar very fine sandy loam	50	20	20	8
	200		22	6
	400		32	6
Amite sandy loam	50	19	17	2
	200		26	5
	400		38	5
Coxville clay	50	12	13	3
	200		13	3
	400		11	4

immediate significance to plant growth. The availability of added soil phosphorus depends upon many factors, particularly the nature of the soil and the type of plant. The problem of correlating the actual utilization of nutrients by plants with the results of soil extraction procedures has been a difficult one. The use of the tracer technique affords a simple method of following the actual plant uptake with physiological amounts of the element, and

TABLE II

RECOVERY IN OAT TOPS OF RADIOACTIVE PHOSPHORUS ADDED TO VARIOUS TYPES OF SOIL

SOIL TYPE	CONTAINER	NUMBER OF CONTAINERS	GROWTH*	ADDED RADIOACTIVE PHOSPHORUS RECOVERED IN OAT TOPS
			gm.†	%
Leon fine sand	Greenhouse jar	6	3.5 ± 0.2	33.0 ± 2.0
Leon fine sand	Lysimeter jar	4	20.2 ± 0.5	32.9 ± 1.3
Hernando fine sand	Greenhouse jar	6	3.9 ± 0.7	16.8 ± 3.5
Amite sandy loam	Lysimeter jar	2	19.6 ± 0.8	13.0 ± 0.4
Coxville clay	Lysimeter jar	2	17.1 ± 1.6	11.3 ± 2.1

* Grams of dry weight of tops.
† The mean value ± the mean deviation.

furnishes a convenient means of comparing the extraction procedure with the biological method under controlled conditions.

In one set of plant experiments glazed greenhouse jars were employed, holding 1000 gm. of soil to which 58 p.p.m. phosphorus as citrate soluble phosphate had been added. About a dozen oat plants were grown in a circle in each jar; when the seedlings were about 2 inches high the appropriate amount of radioactive phosphorus in 10 ml. solution was added to a hole 2½ inches deep punched in the center of the soil area. The jars were watered regularly according to usual greenhouse practice. In other studies 4-gallon glazed lysimeter jars were employed holding 14,000 gm. of soil; the top 4 inches, 7000 gm., had previously been treated to contain 49 p.p.m. phosphorus. About 40 oat plants were grown in each lysimeter jar and the procedure followed as described above.

Radioactive phosphorus was applied February 21 and the plants harvested April 25, 1946. The tops were dried and weighed to give the growth data; samples, usually 2 gm., were ashed with magnesium nitrate according to the usual methods for phosphorus determination. The ash was taken up in hydrochloric acid solution, made to volume, and the activity measured as earlier described.

Table II shows the recovery of radioactive phosphorus in the tops of oats grown on various types of soil. The amount of growth was about the same regardless of the soil type. The percentage recovery from the Leon fine sand was the same, whether the greenhouse or the lysimeter jars were used. With respect to phosphorus recovery the soils ranked in the same order as determined by the chemical and tracer methods; however, the differences were not so great as might have been expected from the values reported in table I. Using the 50-p.p.m. level as the basis of comparison, it was evident that chemical analysis and radioactive phosphorus in equilibrium solution gave the best correlation with plant uptake. With these methods the relative fixing capacities of the Leon fine sand and the Hernando fine sandy loam were in close agreement; this was true also for the Amite sandy loam compared with the Coxville clay. However, a comparison of the Leon and Hernando with the Amite and Coxville showed less differences in fixing capacities when measured by plant uptake than when measured with radioactive phosphorus in equilibrium solution.

The Leon fine sand is of particular interest since it is widespread throughout Florida and has usually been found deficient in phosphorus. About 33% of the added phosphorus was recovered in the oats grown under what might be considered as intensive cropping. Under the experimental conditions reported here it would have been necessary to use only 0.6 micrograms of the phosphorus preparation per jar for adequate measurements, which corresponds to an initial activity of about 0.6 microcuries. This means that field work, leaching studies, or the growing of plant material containing radioactive phosphorus to be fed to animals for metabolism investigations will not be economically prohibitive.

Summary

Convenient procedures for the use of radioactive phosphorus in soil-plant research are described. With a sample having an initial specific activity of 0.95 millicuries per mg. the measurements were sensitive to 0.00015 micrograms phosphorus at 12 days after preparation.

A series of Florida soils showed the same ranking with respect to phosphorus fixing capacity as determined by (a) chemical analysis of equilibrium solution (b) radioactive phosphorus in equilibrium solution (c) radioactive phosphorus in extraction of air dry soil and (d) uptake of radioactive phosphorus by oat plants grown for 63 days.

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RELATIONS BETWEEN CARBOHYDRATE ACCUMULATION AND RESISTANCE OF COTTON PLANTS TO PHYMATOTRICHUM ROOT ROT IN DRY SUMMERS[1]

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(WITH TWO FIGURES)

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As observed by several investigators (**3, 4, 9, 11, 12, 13, 14**), phymatotrichum root rot is notably less severe in dry than in moist summers. Increases have likewise been noted in the numbers of plants dying from root rot after rains and irrigations. The question has not been raised before, however, as to whether the low root-rot mortality of cotton plants in dry seasons is due to moisture conditions unfavorable to the spread of the fungus, *Phymatotrichum omnivorum* (Shear) Duggar, through the soil, or to an associated change in the disease resistance of the plant.

Increased resistance of cotton plants to phymatotrichum root rot has been shown elsewhere by EATON and RIGLER (**2**) to follow increases in the carbohydrate concentrations in the root bark. In that investigation carbohydrate levels were altered by such means as defruiting, partial defoliation, and adjustments in nitrogen supply; the effects of drought on carbohydrate levels and upon root rot were not considered.

The data developed in this paper deal with (1) the effect of drought on the accumulation of carbohydrates and nitrogen in the root bark of cotton plants growing in dry and irrigated field plots, (2) with the effect of drought, in the same experiments, on the severity of phymatotrichum root rot, and (3), by means of laboratory measurements, with the ability of the fungus to grow from a moist substrate into soils with high moisture tensions.

Methods

The concentrations of sugars were measured by the WILDMAN and HANSEN semi-micro procedure (**15**), starch was hydrolyzed with diastase, and hemicellulose with hydrochloric acid. These methods have been reported in greater detail elsewhere (**1**). The procedures followed in the field experiments and in the laboratory measurement of fungus growth are dealt with in conjunction with those results.

Results

CARBOHYDRATE ACCUMULATION AND ROOT ROT IN DRY AND IRRIGATED PLOTS

Field experiments were conducted at the Blackland Substation, Temple, Texas, in 1942 and again in 1944. The experiments included dry and irrigated plots on Houston Black clay in a field that had shown much root rot in previous years.

[1] Approved as Technical Paper no. 959 of the Texas Agricultural Experiment Station.

The 1942 experiment was looked upon as exploratory and was laid out in only two blocks, each with four plots. The four treatments were as follows: (1) irrigated, (2) irrigated and fertilized, (3) dry, and (4) dry and fertilized. Five acre-inches of water were applied to the irrigated plots on July 10 and again on August 10. The rainfall was negligible between the middle of June and August 12, but on the latter date there was a rain of 0.48 inches, and on August 16 there was a rain of 1.56 inches. Rains of 0.61, 0.13, 0.52, and 0.11 inches fell on August 19, 24, 30, and 31, respectively. During the first ten days of September rainfall amounted to 6.35 inches. The fertilized plots received 800 pounds per acre of 9–6–3 commercial fertilizer placed beneath the drill rows at time of planting (May 4).

The plants for root-bark samples were collected between 10:00 A.M. and

TABLE I

MEANS OF CARBOHYDRATES, TOTAL NITROGEN, AND MOISTURE CONCENTRATIONS IN ROOT BARK (ONE DATE) AND PLANTS KILLED BY ROOT ROT (ONE DATE) IN UNFERTILIZED WET AND DRY PLOTS IN 1942*

Item	Wet		Dry	
	Unfertilized	Fertilized	Unfertilized	Fertilized
	%	%	%	%
Root-bark constituents:				
Hexoses	0.56	0.68	1.11	1.05
Sucrose	1.02	1.07	1.53	1.98
Starch	0.84	0.86	1.03	1.16
Total CHO	2.42	2.61	3.41	4.20
Hemicellulose	9.12	9.63	9.79	10.45
Total nitrogen	0.130	0.152	0.136	0.201
Moisture	79.0	78.0	75.4	75.4
	gm.	*gm.*	*gm.*	*gm.*
Weight tap roots	17.1	21.7	11.2	12.2
	mean %	*mean* %	*mean* %	*mean* %
Plants killed by root rot: on Oct. 1	50	52	27	21

* Analytical data expressed as percentage of fresh weight.

11:00 A.M. on the morning of August 17 after irrigation of the irrigated plots on August 10 and after rains of 0.48 inches and 1.56 inches on August 12 and 16, respectively. At no time did the plants in the dry plots show any marked degree of wilting, but it may be noted that the two irrigations resulted in a large increase over the dry plots in the weight of the tap roots (table I). Each of the 16 samples for carbohydrate analysis was a composite of the root bark from 15 uninfected plants selected at random in the upper and lower halves, respectively, of each of the 8 plots.

Although samples were collected following a day of rain, the concentrations of hexoses, sucrose, and starch were all higher in the dry than in the irrigated plots. Drought resulted in an increase of 51% in the sum of these carbohydrates (table II). Fertilization also increased carbohydrate

concentrations, but it is probable that part of the increase is attributable to differences in moisture supply; *i.e.*, with the same amount of rain and irrigation, the fertilized plants were larger as shown by the 18% increase in weight of tap roots. The slightly higher concentrations of hemicellulose found in the plants from the dry plots disappear if the data be computed to a residual dry weight basis. There were twice as many plants killed by root rot in the irrigated as in the dry plots on October 1 (table I) and similar differences were found also on September 1 and 15.

The 1944 experiment was undertaken on a more comprehensive scale than the one in 1942, and the summer was more favorable for it. There were 16.75 inches of rain between May 1 and June 12, but thereafter there were no rains of consequence until the period of August 27 to 30 when 1.91

TABLE II

PERCENTAGE CHANGE AS A RESULT OF DROUGHT AND MINERAL FRETILIZER ON ROOT-BARK CONSTITUENTS AND PLANTS KILLED BY ROOT ROT IN 1942—FACTORIAL SUMMARY

ITEM	DROUGHT*	FERTILIZER†
	%	%
Root-bark constituents:		
Hexose	74§	4
Sucrose	68§	28‡
Starch	29‡	11
Total CHO	51§	17‡
Hemicellulose	7	6
Total nitrogen	19§	3‡
Moisture	− 3§	− 1‡
Weight	−40§	18§
Plants killed	−53‡	− 5

* Determined by the difference between the means of wet and dry treatment results.
† Determined by the difference between the means of the unfertilized and fertilized treatment results.
‡ Statistically significant by odds of 19 to 1.
§ Statistically significant by odds of 99 to 1.

inches fell. On September 6 and 7 there was another rain of 1.68 inches. Starting on July 14 the wet plots were irrigated with one acre-inch of water weekly. Fertilization consisted of 6.4 tons of oat straw (air-dry basis) plus 100 pounds of inorganic nitrogen per acre. These materials were disked into the soil prior to planting on May 13. The four treatments: (1) irrigated, (2) irrigated plus manure, (3) dry, and (4) dry plus manure, were randomized in each of ten blocks. Dead-plant counts were made at intervals of about 15 days starting in mid-July. The means of the percentages of dead plants on July 31, August 14, September 1 and September 15 are shown (table III). On September 15 there were, respectively, 67% and 76% of the plants dead in the unfertilized and manured irrigated plots, and 13% and 10% in the corresponding dry plots. The soil-moisture samples were taken at a depth of 6 to 8 inches on the day before the irrigations of July 27, August 3, 10, 18, and 24; the root-bark samples were collected between 10:00 A.M. and 11:00 A.M. on each of four dates during the fruiting

period (fig. 1). Each collection was in triplicate for each treatment, with one uninfected root from each of ten plots making a single sample.

The total-carbohydrate concentrations as found on each of the four dates in 1944 (fig. 1) are summarized as separate constituents (table III). Consistent with the earlier results, the hexoses, sucrose, and starch concentrations were higher in the dry than in the irrigated blocks. The greatest effect of drought was found in starch concentrations. Little effect of manuring is reflected in the hexoses or sucrose sugars, but manuring resulted in large decreases in starch, both in the wet and dry plots.

Drought greatly increased the concentrations of total nitrogen in the root bark (table IV). Manuring, however, had only minor effects on nitrogen

TABLE III

MEANS OF CARBOHYDRATES, TOTAL NITROGEN, AND MOISTURE CONCENTRATIONS IN ROOT BARK (FOUR DATES) AND PLANTS KILLED BY ROOT ROT (FOUR DATES) IN UNFERTILIZED AND MANURED WET AND DRY PLOTS IN 1944*

ITEM	WET		DRY	
	UNFERTILIZED	MANURED	UNFERTILIZED	MANURED
	%	%	%	%
Root-bark constituents:				
Hexose	1.00	1.02	1.18	1.21
Sucrose	1.69	1.72	2.13	2.07
Starch	1.27	0.95	2.46	1.79
Total CHO	3.96	3.69	5.77	5.08
Total nitrogen	0.242	0.268	0.365	0.359
Moisture	74.6	75.4	70.1	71.0
	gm.	*gm.*	*gm.*	*gm.*
Weight per bark	11.8	13.3	8.4	9.0
	mean %	*mean* %	*mean* %	*mean* %
Plants killed by root rot	38	42	10	6

* Analytical data expressed as percentage of fresh weight.

levels in the experiment, and the responses were greater in the dry than in the irrigated plots.

The relations found between the severity of root rot and carbohydrate concentrations in the root bark are shown (fig. 1) by graphing carbohydrates and root rot, the latter being expressed as the percentages of living plants which died during the successive periods of the experiment. Root rot first makes its appearance in cotton fields in the summer as scattered dead or wilted plants. As these centers of infection, and new ones, enlarge concentrically, it is the plants on the peripheries that are subject to attack. As the peripheries become longer, increasing numbers of live plants are in positions to be attacked, but as the margins of adjacent dead areas come together, the trend in the number of plants subject to attack may be reversed.

Irrespective of whether the spread of the fungus from plant to plant is primarily along overlapping roots or by the growth outward of the fungus

through the soil, cotton roots provide the organic substrate, and the spread of the fungus is largely limited by root spread. The rate of spread of the disease is thus dependent, among other conditions, upon the availability of the organic substrate provided by the cotton roots and the suitability of this root material for the fungus. The measurement of this suitability (disease resistance) can be made fairly direct in the case of plants that are growing in pots where the days from inoculation to death can be measured; measure-

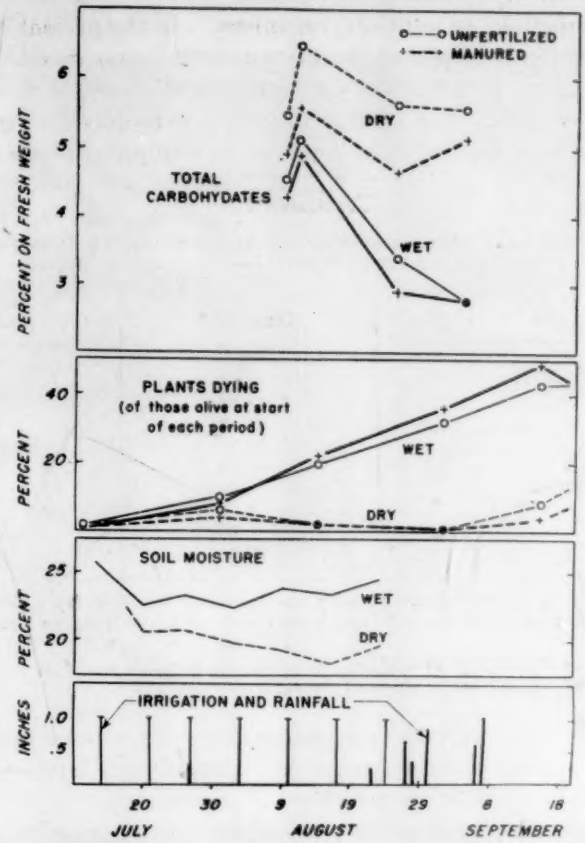

FIG. 1. Carbohydrate levels, severity of phymatotrichum root rot, soil moisture, rainfall, and irrigation during the 1944 experiment.

ment cannot be so direct in the field, however, for there are no means for determining the time lapse between infection and death.

Notwithstanding these difficulties of measurement, a general inverse relation is shown (fig. 1) between differences in carbohydrate levels in the plants and differences in root rot between (1) the unfertilized irrigated and manured irrigated plots and (2) between these two treatments and the two dry treatments considered collectively. There was as little root rot in the dry plots with manure as in those without manure, but there were much

lower carbohydrate levels in the former. The effects of the manure can be considered in this connection.

Various investigators have found manuring to be one of the most effective means of controlling root rot. As reported by KING and LOOMIS (**6**) a 10-ton application of manure to irrigated plots in Arizona resulted in much lower percentages of death until mid-August, but later the rate of spread was as great in the manured as in the check plots; presumably by this time the decomposition of the manure had proceeded to the point that it no longer supported competitive or antibiotic organisms. In the present experiments the data show no evidence that the 6.5 tons of manure provided antibiotic protection against root rot in the wet plots, but this was not necessarily the case in the dry plots. The manure decomposed rapidly in the wet plots, but a good bit of it was still in evidence in the dry plots in late September.

TABLE IV

PERCENTAGE CHANGE AS A RESULT OF DROUGHT AND MANURE ON ROOT-BARK CONSTITUENTS AND ROOT ROT IN 1944—FACTORIAL SUMMARY

ITEM	DROUGHT*	MANURE†
	%	%
Root-bark constituents:		
Hexose	18.8§	2.8
Sucrose	23.5‡	0.0
Starch	91.0§	−26.3§
Total CHO	41.9§	− 9.9
Total nitrogen	42.0§	3.2
Moisture	− 6.1§	1.4§
Weight	−31.0§	10.8§
Root rot	−80.0§	0.0

* Determined by the difference between the means of wet and dry treatment results.
† Determined by the difference between the means of unfertilized and manured treatment results.
‡ Statistically significant by odds of 19 to 1.
§ Statistically significant by odds of 99 to 1.

In other words the lower root-rot mortality in the dry-manured plots than in the dry plots without manure—relative to carbohydrate levels—could have been due entirely to the antibiotic effects of the manure.

There remain for consideration relations between the rates of plant death and variations in carbohydrate levels as found at the four sampling dates. The carbohydrate levels found on August 9 and 11 were higher than subsequently and there was a correspondingly lower mortality rate in the irrigated plots in early August. It is doubtful, however, that any significance can be attached to this relationship. The four sets of samples were sufficient to establish the relative levels of carbohydrates under the four treatments, but the four collection dates do not necessarily reflect variations in carbohydrate levels between sample collections. Furthermore, the method employed for representing resistance to root rot by rate of plant deaths cannot be expected to reflect accurately day-to-day changes in resistance.

GROWTH OF FUNGUS INTO SOILS OF VARIED MOISTURE CONTENT

Concurrently with the establishment of *Phymatotrichum omnivorum* on and in a cotton root, hyphal wefts grow forward along the root, etching the epidermis some distance in advance of actual invasion. The root-rot fungus probably makes its most rapid advance from plant to plant along roots, but it is also certain that there is a substantial outward growth through the soil that results in the establishment of the fungus in nearby roots. Irrespective of the main avenue of advance, it is reasonable to expect that the growth of

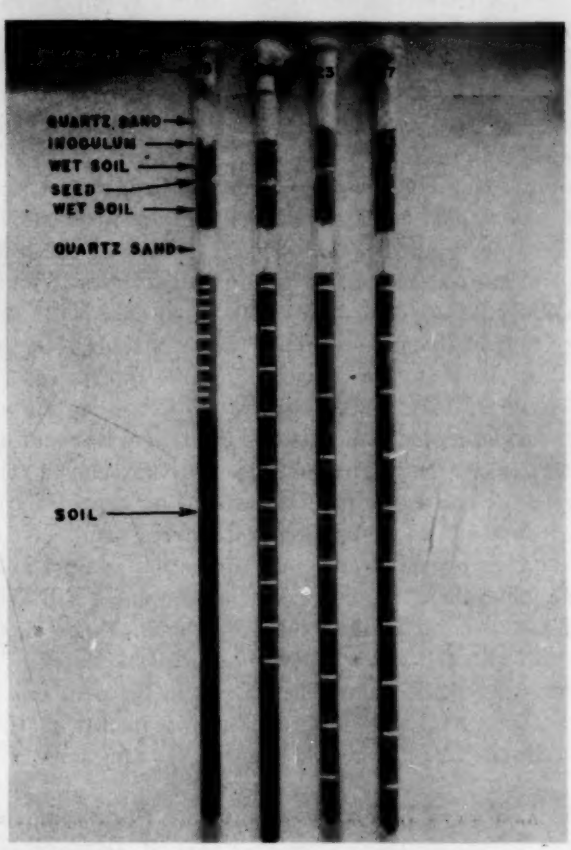

FIG. 2. Daily growth rate of *P. omnivorum* from a moist substrate (across a quartz-sand bridge) into soils of varied moisture content.

this fungus, unless it be effectively protected against evaporation by a coating of suberin-like materials or by a physico-chemical mechanism for water retention, would be greatly influenced in its growth by the tension of the soil moisture. If in dry soils the root-tissue substrate provides most or all of the moisture required for growth, and water loss from the mycelium to the soil occurs very freely, then water movement from the tissue substrate through the strands and hyphae would have to proceed with great rapidity.

In the light of the cellular structure of this fungus, it is difficult to understand how a very rapid movement of water could occur. In any event a need is indicated for evidence of the ability of the fungus to grow from a moist substrate into soils with high moisture tensions.

An experiment was undertaken for the purpose of measuring the rate of growth of the fungus from a moist substrate into soils of varied moisture content. Both the means adopted for the measurements and representative results are illustrated (fig. 2). The importance of the information relative to one of the major considerations of this paper is fairly obvious. If the growth of this fungus be sharply restricted as soils become dry, even though provided with a source of moisture in root tissues, there could be little progress of the disease. This would be the case whether or not the plant, through the consequences of its own reactions to low soil moisture, had developed a high degree of resistance.

The Houston Black clay employed for the experiment had a moisture equivalent of 30.9%. Aliquots of this soil were adjusted to four moisture percentages by water additions and sieving. Each of the soils was slowly added, with a substantial loss of moisture, to glass tubes 15 mm. in diameter and approximately 60 cm. long to a depth of 43 cm., while the bottoms of the tubes were tapped sharply on the surface of a table. On top of the soil there were added in sequence: 4 cm. of coarse quartz sand (to prevent capillary movement), 3 cm. of dry soil, 25 moistened sorghum seeds, 3 cm. of dry soil, 10 sorghum seeds, and lastly 4.5 ml. of water and a cotton plug. After standing upright overnight the tubes were autoclaved for one hour on two successive days and inoculum then added on top of the upper seeds. The tubes were finally filled to the plugs with sterile sand. Neither the sand nor the cotton plugs provided adequate protection against evaporation; at the end of the experiment the outer half of the moist soil above the sand bridge was found to be drier than the inner half. Some vapor movement from the wet soil into the lower soil was likewise indicated. The approximate initial moisture percentages for the soil in place in the tubes are the rounded values found for the 7-day to 9-day segments at the end of the growth period (table V). There were four tubes for each of the four moisture levels.

Sterile methods were necessary to prevent the decomposition of the sorghum-seed substrate by organisms other than *P. omnivorum*. The tubes were kept on their sides in a transfer room at 28° C. during incubation and while the daily growth measurements were being recorded. Measurements were referenced to a crayon mark that located the position of the fungus in the soil on the first morning after it had grown through the sand bridge. This date was not the same for all tubes.

There was little difference in the rate of growth of the fungus into soils A and B with respectively 27% and 23% of moisture (table V). The growth rate in the C tubes, which contained initially 20% moisture was 70% of that in the A and B tubes. Growth in the D tubes, with 15% initial moisture, was 23% as great as in the A and B tubes.

The "first permanent wilting point" and the "ultimate wilting point" as determined by the method of FURR and REEVES (5) were for this soil 22.2 and 18.1, respectively. Knowing these values it is possible to orient roughly the soil moisture percentages of the soil used in the various tubes in terms of soil moisture tensions. As determined by the pressure-membrane method, RICHARDS and WEAVER (10) found the tensions for 24 of the Furr-and-Weaver soils at "first permanent wilting" to range from 5 to 13 atmospheres with 14 of these soils in the 7 to 9 atmosphere range. At "ultimate permanent wilting" 17 out of 24 of the soils fell within the 20 to 30 atmosphere range.

It is evident that the outward growth of the fungus from a moist substrate was not affected by soil-moisture tensions (the B tubes with 23% moisture) approaching, if not exceeding, 5 atmospheres. Growth was retarded only 30% by tensions probably in the range of 10 to 20 atmospheres in the C tubes that had 20% moisture. The fungus was able to make a substantial growth into a soil with moisture tensions that may have been well in excess of 30 atmospheres—the D tubes with 15% moisture.

TABLE V

GROWTH OF *Phymatotrichum omnivorum* INTO SOILS OF VARIED MOISTURE CONTENT WHEN SUPPORTED BY SORGHUM SEED IN A MOIST-SOIL SUBSTRATE

TUBES	INITIAL MOISTURE CONTENT OF SOILS IN FILLED TUBE	MEAN GROWTH OF FUNGUS			SOIL MOISTURE		
		SECTION 0–3 DAY	SECTION 4–6 DAY	SECTION 7–9 DAY	SECTION 0–3 DAY	SECTION 4–6 DAY	SECTION 7–9 DAY
	%	*mm./day*	*mm./day*	*mm./day*	%	%	%
A	27	44	44	42	28.6	27.6	27.0
B	23	45	41	40	25.9	23.2	23.0
C	20	31	32	30	20.7	19.8	20.3
D	15	9	10	10	18.4	16.3	15.0

The experiment provides no explanation of the ability of *P. omnivorum* to retain or move its water and grow into the drier soils. This ability is of special interest because of the fact that the new hyphae had diameters in the order of 0.01 mm. The single hyphae advanced ahead of strand formation by about one day, and the largest strands and occasional sclerotia developed three or four days later.

The foregoing results are largely in accord with earlier measurements by ROGERS (11) who placed soil (of the same type and collected from near where the present soil was obtained) in jars and inserted pieces of infected cotton roots. A substantial growth of the fungus occurred in soil with 15% moisture, and 25% moisture appeared to him to be near the optimum.

The fact that *P. omnivorum* is found to possess a remarkable ability to grow from a moist substrate into soils with high moisture tensions bears on the observed low incidence of the disease in the dry plots. A greater death rate of plants in these plots should have occurred if the direct effects of low

soil moisture on the growth of the fungus had been the only factor retarding its spread—either along roots or from the roots of one plant to those of another. In other words, a plant resistance factor is indicated as having been responsible for a large part of the low root-rot mortality in the unfertilized dry plots of the experiments and, more broadly, in dry years generally.

Discussion

In the investigation (**2**) that preceded this one it was shown that marked changes occurred in the numbers of root-surface saprophytes as carbohydrate levels in the root bark were increased; at the higher carbohydrate levels the numbers of some types of organisms were increased and others were decreased. Inasmuch as *P. omnivorum* makes an excellent growth on high carbohydrate substrates under sterile conditions, it was concluded that the increased resistance was probably the result of the competition or increased antibiotic activities of one or more of these root-surface saprophytes and the parasitic activity of this root-rot fungus was demonstrated by means of experiments with maize. After inoculation with *P. omnivorum*, maize plants growing on sterile sand-bentonite substrates were rapidly attacked and killed, whereas the roots of maize plants on otherwise similar but nonsterile substrates remained healthy even though paralleled for long distances by strands of the fungus.

Data bearing directly on the effects of drought on the root-surface microfloras of cotton plants are not available, but MITCHELL, HOOTON, and CLARK (**7**) have presented results which show that major changes occurred in the numbers of various root-surface organisms with the advance of a summer at Greenville, Texas, and further, that some of these variations were obviously due to factors other than the aging of the cotton plants.

The present data extended to the effects of drought, the earlier showing (**2**) that increased resistance to phymatotrichum root rot results when carbohydrate levels are increased by such diverse means as withholding nitrogen and defruiting and debranching both with and without increased light intensities. The possibility remains that the changes effected in carbohydrate levels by various means altered or accompanied changes in some other constituent of the root bark which was more immediately responsible, either directly or *via* altered antibiotic activity, for changes in resistance. It was shown in previous work, however, that the level of total nitrogen in the root bark apparently is not involved.

The equilibriums between sugars and starch are rarely uniform either throughout the daily cycle or from day to day. Neither in this paper nor in the previous one (**2**) has any great effort been made to conclude which of the determined carbohydrate components might bear the most direct relation to changes in resistance. In a field experiment somewhat higher correlation between the number of dead plants and the sum of the sugars was obtained than between resistance and total carbohydrates. Tables II

and VII of the previous paper (**2**) in which relations between the carbohydrates and root-surface saprophytes of healthy roots were considered, present evidence that sugars correlated better than total carbohydrates with the changes in the numbers of some, but not all, of the root-surface saprophytes. Prior to actual invasion of the fungus into the root tissue it would seem that there might be a more direct effect of sugars on the root-surface microflora than of total carbohydrates by reason of possible equilibriums between interior and exterior sugar concentrations. During decomposition, however, after invasion has occurred, the hydrolysis of less labile carbohydrate and other tissue components is actively in progress. The growth of *P. omnivorum* is primarily dependent upon the substrate provided by this decomposing mass of tissue, and during the decomposition many constitutents contribute to the growth of the fungus and affect also the nature and activities of the competing saprophytic microflora.

Summary

Phymatotrichum root rot of cotton has been observed for many years to be much less severe in dry than in moist summers. This investigation was directed toward the question of whether low plant mortalities during periods of drought represent an inability of the fungus, *P. omnivorum*, to grow outward from the root substrate to infect neighboring plants or whether the disease resistance of the plants is altered by drought.

Laboratory measurements are presented which show that *P. omnivorum* when supplied with a moist substrate makes almost as rapid a growth outward into soils with moisture percentages within the wilting range as into soils with higher moisture percentages.

During two summers it was found that root rot was far more severe and carbohydrate concentrations in the root bark of cotton plants were much lower in irrigated than in dry plots. The inverse parallelism found between the severity of root rot and carbohydrate concentrations, as influenced by moisture supply, is in agreement with results of previous investigations in which carbohydrate levels were altered by such diverse means as limiting the nitrogen supply and debranching and defruiting. The data are discussed briefly in relation to changes found previously in the numbers of various root-surface saprophytes when carbohydrate levels were increased, and the possible bearing of stimulated antibiotic activity on the results obtained.

The writers are indebted to NEIL E. RIGLER for his collaboration in part of the investigation.

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BRIEF PAPERS

INVERSE CORRELATION BETWEEN RUBBER HYDROCARBONS AND INSOLUBLES IN TOTAL SOLIDS OF LATEX FROM *HEVEA BRASILIENSIS*

W. S. Stewart and R. W. Hummer[1]

(WITH ONE FIGURE)

Received September 20, 1946

Recent studies of the total solids in latex from the rubber-bearing plant *Cryptostegia grandiflora* indicated that a linear inverse correlation existed between the percentages of rubber hydrocarbons and a crystalline substance isolated from the insolubles (**1**). This stimulated a similar investigation into the relationship between rubber hydrocarbons and insolubles in the total solids of latex from *Hevea brasiliensis*.

Accordingly, thirty-four samples of latex from *H. brasiliensis* were collected and analyzed for rubber hydrocarbons and insolubles. In these samples a wide distribution of rubber hydrocarbons from low to high percentages was intentionally sought by bleeding petioles and stems of young seedlings as well as trunk-tapping mature trees in production. Except for six collections, the latex was air-dried on a glass sheet immediately upon exudation. When it was dry enough tó allow handling, it was removed from the sheet and further dried to constant weight at 60° C. The dried latex, called "latex total solids," formed the basis on which percentage composition was calculated. The six latex samples not dried on a sheet of glass immediately after exudation were "rubber biscuits" formed in commercial latex collecting cups as remnants of dried latex from the previous tapping. All latex samples were collected from April, 1944, to March, 1945, from two widely separated locations in Mexico and from two locations in Costa Rica. Because of the low latex yields from petioles and seedlings these samples were composite collections of from five to forty individuals. The trunk latex samples from producing trees of seedling origin were analyzed on an individual trees basis. This allowed for more variation between samples than if they had been composited. The considerable diversity represented by the collections is shown (table I).

Duplicate analyses of the total solids for rubber hydrocarbons were made by a bromination technique while insolubles were determined gravimetrically (**1**). By these methods, rubber hydrocarbons and insolubles were thus both directly determined. This is an important point since these two fractions together constitute a large percentage of the total solids. If, therefore, either of them was determined indirectly by difference, an ap-

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TABLE I

ANALYSES OF LATEX TOTAL SOLIDS FROM *Hevea brasiliensis*. UNLESS INDICATED OTHERWISE, LATEX WAS COLLECTED BY TRUNK TAPPING. TRUNK LATEX SAMPLES ARE FROM INDIVIDUAL TREES; ALL OTHER SAMPLES ARE COMPOSITE YIELDS OF FROM 5 TO 40 INDIVIDUALS

DATE	LATEX SOURCE AND TREE AGE	LOCATION	LATEX ANALYSES		
			RUBBER HYDROCARBON	INSOLUBLES	UNDETERMINED (DIFFERENCE)
			%	%	%
Mar. '45	Leaf petioles	El Palmar, V. C., Mexico	47.3	36.7	16.0
" "	½–¾ inch dia. green lat. br.	" " " " "	49.3	39.4	11.3
May '44	Mature petioles, 1½-yr. seedling	Gomez Farías, Tps., Mex.	49.4	34.3	16.3
Mar. '45	½–¾ in. gr. side branches	El Palmar, V. C., Mexico	52.3	34.0	13.7
" "	Leaf petioles on 2-yr. tree	" " " " "	54.0	33.0	13.0
July '44	Stems, seedlings	Turrialba, Costa Rica	54.6	33.0	12.4
Aug. '44	Mature petioles on 10-mo. seedl.	El Palmar, V. C., Mexico	65.9	22.1	12.0
July '44	Petiole young leaf, clone GV 21	" " " " "	67.3	21.3	11.4
Apr. '44	Stems, 15-month seedlings	" " " " "	68.6	17.4	14.0
July '44	Mature petioles, 3-yr. seedling	" " " " "	69.7	19.0	11.3
" "	Petiole young lf., clone GA 49	" " " " "	70.5	18.7	10.8
Mar. '45	Two years, group B	" " " " "	70.7	18.0	11.3
" "	" " " A	" " " " "	71.5	13.3	15.2
May '44	Estimated 2 years	Gomez Farías, Tps., Mex.	72.0	10.0	18.0
" "	" 1½ "	" " " "	73.3	10.6	16.1
July '44	Clone GA 49, stem 1 inch dia.	El Palmar, V. C., Mexico	73.7	16.7	9.6
Mar. '45	Two years, group A	" " " " "	75.2	13.3	11.5
" "	" " " B	" " " " "	75.6	20.3	4.1
July '44	Clone GV 21, stem 1 inch dia.	" " " " "	76.9	12.9	10.2
Apr. '44	In production, estimated 20 yr.	" " " " "	77.7	4.7	17.6
July '44	Plantation A, 3 years	" " " " "	77.8	9.8	12.4
" "	Seedling, 1½ years	" " " " "	78.3	9.8	11.9
" "	Plantation B, 3 years	" " " " "	78.4	10.8	10.8
" "	In production, seedling origin	Cairo, Costa Rica	81.5	1.4	17.1
" "	" " " "	" " "	82.0	1.7	16.3
" "	" " " "	" " "	82.2	2.2	15.6
" "	" " " "	" " "	82.8	4.0	13.2
" "	" " " "	El Palmar, V. C., Mexico	83.0	3.3	13.7
" "	" " " "	" " " " "	83.2	5.4	11.4
" "	" " " "	" " " " "	84.2	2.8	13.0
" "	" " " "	Cairo, Costa Rica	84.3	0.6	15.1
" "	" " " "	" " "	84.9	1.6	13.5
" "	" " " "	" " "	85.7	0.4	13.9
" "	" " " "	" " "	89.0	1.1	9.9

parent correlation could be indicated though actually non-existent. According to these analytical methods, insolubles in total solids are defined as substances which are insoluble in either acetone or benzene; and rubber hydrocarbons are benzene-soluble substances which form bromides insoluble in 95% ethyl alcohol.

From these analyses, it was found that the percentage of rubber hydrocarbons was inversely correlated with the insolubles (table I and fig. 1). The correlation coefficient between percentage of rubber hydrocarbons and

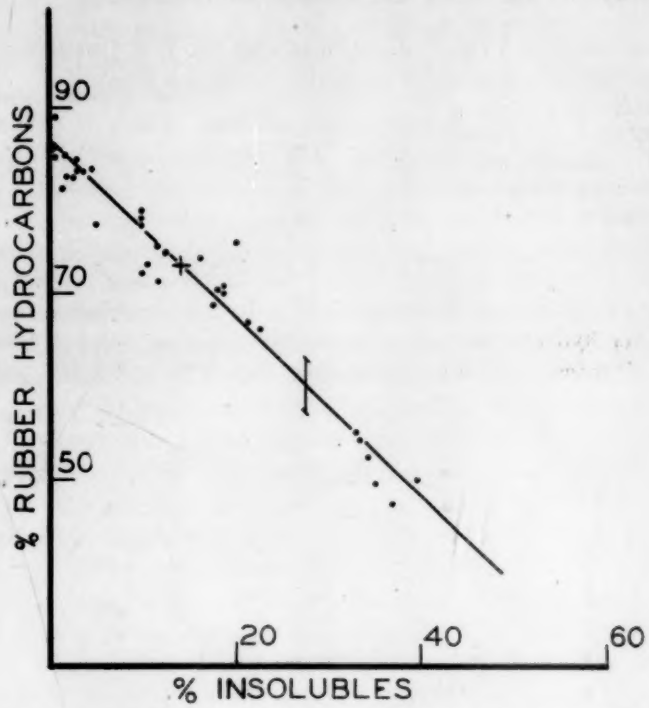

FIG. 1. Inverse correlation between percentage of rubber hydrocarbons and insolubles in total solids of latex from *Hevea brasiliensis*. Regression line with standard error calculated from 34 analyses. Cross shows average point.

insolubles was – 0.970. Since these analyses appear to represent a straight line relationship, it was possible, by using the method of least squares for the computation of regression averages, to calculate the equation:

$$R\% = 86.6 - 0.972\ I\%$$

where, R = rubber hydrocarbons and I = insolubles. The line defined by this equation (fig. 1) has intercepts of 86.6 and 89.2 on the rubber hydrocarbons and insolubles axes, respectively. The standard error of the estimate was $\pm$ 2.8%. The regression coefficient, – 0.972, is significant at the 1% level. The corresponding regression coefficient for a similar relationship in latex from *C. grandiflora* was – 0.809.

The significance of the inverse correlation between rubber hydrocarbons and insolubles in latex from *H. brasiliensis* is not yet understood. Isolation and identification of the substance, or substances, in the insolubles which cause the correlation may indicate if this relationship is caused by the presence of rubber hydrocarbon "precursor" in the insolubles or by some other means. It is clear that the correlation affords a basis for further physiological studies on latex rubber formation. The fact that a similar relationship exists in the latex from rubber-producing plants of two different botanical families may indicate that it is of a fundamental nature.

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THE FORMATION OF CHLOROPHYLL *A* IN ETIOLATED OAT SEEDLINGS

RICHARD H. GOODWIN AND OLGA V. H. OWENS

(WITH ONE FIGURE)

Received October 4, 1946

The ratio of chlorophyll *a* to chlorophyll *b* has been determined for the green tissues of many species of plants. Chlorophyll *a* usually lies between 67% and 78% for the normal green tissues of higher land plants (**2, 10, 11**).

In etiolated tissues, however, chlorophyll *a* has been reported to be formed much more rapidly than *b*, when the plants are first exposed to light, regardless of the spectral region employed (**8**). Although not specifically commenting on this interesting phenomenon, FRANK (**3**) has recently published spectrophotometric data indicating that the same thing holds true for completely dark-grown seedlings of *Avena byzantium* var. *sativa*, after five-hour exposures to light. It is possible that this early rapid production of chlorophyll *a* may be correlated with the initial presence in dark-grown plants of a relatively higher percentage of "protochlorophyll *a*" than of "protochlorophyll *b*," a condition which reportedly exists in pumpkin seeds (**9**).

In studies on the production of chlorophyll in *Avena sativa* (**5**), the authors have observed an almost complete absence of chlorophyll *b* in chlorophyll extracts obtained from plants irradiated for two or three hours with visible light. Husked seeds were laid out on wet absorbent cotton and germinated in complete darkness under controlled conditions of temperature and humidity. After two and a half days, some of the seedlings were irradiated for two hours with monochromatic blue light (4358 Å) isolated with a Corning polished glass filter #5113, 2 mm. thick, a 5-cm. layer of 1.2% $CuSO_4$, and a 5-cm. layer of 0.4% quinine hydrochloride in N/5 HCl. The transmissions of the liquid filters have been reported elsewhere (**1**). Radiation intensity at the level of the plants was approximately 5.5 ergs per mm.^{2} per second. The shoots of the irradiated seedlings and of dark-grown controls were then removed just below the coleoptilar node and ground in pure acetone. The acetone extract was centrifuged and the pigments transferred to ethyl ether in a separatory funnel. The acetone was removed by successive washings with distilled water to which a trace of calcium carbonate had been added. Extractions were carried out in very dim light and the extracts stored in the dark for two days over solid carbon dioxide, until spectroscopic studies could be made.

Absorption spectra were obtained on an automatic recording spectrophotometer (**6**),[1] with the monochromator slit width set at 100 Å. Figure 1 shows the relative absorption per shoot of extracts from completely dark-

[1] We are indebted to DR. ARTHUR C. HARDY of the Physics Department of the Massachusetts Institute of Technology for placing this instrument at our disposal and to DR. S. Q. DUNTLEY for running the absorption curves.

grown plants (curve A) and from irradiated plants (curve B) in the spectral region not obscured by carotenoid absorption. The differences in these curves are due chiefly to the synthesis of chlorophyll during the two-hour exposure. The two absorption maxima for the dark-grown extract probably

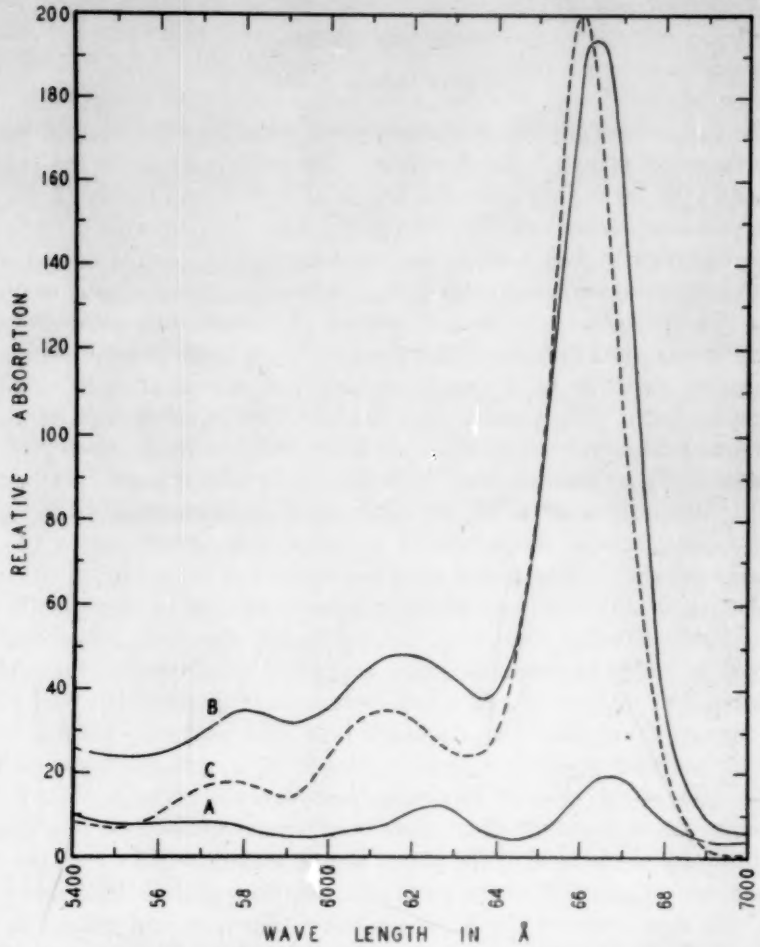

FIG. 1. Absorption spectra of ether extracts of Avena shoots and of pure chlorophyll *a* in ether. Curve A: extract from 429 dark-grown plants; Curve B: extract from 458 plants exposed for two hours to monochromatic blue (4358 Å) light at a radiation intensity of approximately 5.5 ergs/mm.²/sec. The plants in the two groups were of comparable size; the data are expressed as relative absorption per plant. Curve C (broken line): pure chlorophyll *a* (4).

represent the absorption of "protochlorophyll" (6250 Å) (7) and of chlorophyll *a* (6670 Å). The positions of three of the absorption maxima for the extract from irradiated plants at 6640 Å, 6180 Å, and 5800 Å are in close agreement with those for pure chlorophyll *a*, although there appears to be a shift toward the red of about 40 Å. The absorption spectrum of pure

chlorophyll *a*, isolated from spinach by the senior author (**4**) following the method of ZSCHEILE and COMAR (**12**), was obtained on the same spectrophotometer and is shown for comparison in figure 1 (broken curve C). The maxima for this preparation at 6600 Å, 6140 Å, and 5760 Å coincide with those previously reported (**12**).

There is no shoulder in the absorption curve for the extract from irradiated plants at 6425 Å, the region of the chlorophyll *b* absorption maximum (**12**), which would be expected, if appreciable quantities of this component were present. To check whether traces of chlorophyll *b* might be present, another acetone extract from 270 plants irradiated for three hours was centrifuged, and the pigments transferred to petroleum ether (b.p. 35° to 45° C.). The petroleum ether solution was washed repeatedly with distilled water to which a trace of calcium carbonate had been added, dried over anhydrous sodium sulphate, and filtered onto a small adsorption column of powdered sucrose 2 cm. in diameter and 15 cm. high. The chromatogram, which was developed for three-quarters of an hour with petroleum ether, showed but a single blue zone above the carotenoids, no green chlorophyll *b* zone being in evidence. The chlorophyll band was mechanically removed and the pigment eluted with 10 ml. of ethyl ether. Two milliliters of the ether extract were diluted with 18 ml. of acetone, and the fluorescence of the solution, when irradiated with blue (4358 Å) and violet (4047 Å) light was measured on a Klett fluorimeter-colorimeter.[2] The ratio of the fluorescence in the violet to that in the blue was 0.175. This value is nearly that obtained for pure chlorophyll *a* (0.182 ± 0.012), and not that obtained for chlorophyll *b* (0.051 ± 0.001). Details of the fluorescent behavior of chlorophylls *a* and *b* and mixtures of these two components will be given elsewhere (**4**).

It is concluded that virtually no chlorophyll *b* is formed in oat plants during the first three hours of exposure to blue light.

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NOTES

The Boston Meeting.—The twenty-first annual meeting of the American Society of Plant Physiologists was held in Boston, December 27–30, 1946. Five joint sessions of the American Society of Plant Physiologists and the Physiological Section of the Botanical Society of America were held. Sixty-three papers were presented in addition to the three symposia. One symposium was given at each of the following meetings: Section G of the A.A.A.S. on Friday, December 27, Section O and the A.S.H.S. on Saturday, December 28, and Section O on Monday, December 30. The meetings were all well attended and the papers were of more than usual interest. The Saturday symposium on plant respiration was especially well received by a large audience. At the annual meeting of the Council of the American Association for the advancement of Science held in Boston, Dr. Walter F. Loehwing was elected Vice President of the A.A.A.S. and Chairman of Section G for Botanical Sciences for 1947.

The Plant Physiologists' Dinner with Dr. B. M. Duggar presiding was held at the Vendome Hotel on Friday evening, December 27 and was attended by 152 persons. The retiring president's address by Dr. Paul J. Kramer was entitled, "A Viewpoint for Plant Physiologists," in which he stressed the importance of cooperation between research workers in fundamental and applied physiology. Recipients of the Charles Reid Barnes Award and the Stephen Hales Award were announced.

An Executive Committee meeting preceded the business session of the Society held on Monday afternoon, December 30 in order to prepare the agenda for presentation. Several constitutional changes relative to corresponding members and the Program Committee were approved for presentation to the membership in March by mail ballot. These concern the privilege of corresponding members to receive the journal, as well as the bulletins, without charge. It was also recommended that the Secretary act as Chairman of the Program Committee. Questions as to the time and place preferred for the next meeting of the Society, as well as to membership reaction to present and proposed National Science Foundation bills will be presented in the mail ballot.

The Stephen Hales Award.—Dr. Burton Edward Livingston, Professor Emeritus of plant physiology at Johns Hopkins University, was awarded the Stephen Hales Award. Dr. Livingston was born February 9, 1875 in Grand Rapids, Michigan. He received the B.S. degree from the University of Michigan in 1898 and the Ph.D. from the University of Chicago in 1901. Since his graduation Dr. Livingston has been active in the field of plant science, not only as an instructor and research worker, but also as a leader in many of the professional organizations of the country. He was elected to the first Barnes honorary life membership of the American Society of

Plant Physiologists, and has served in the Society as its President, member of the Executive Committee, and member of the Editorial Board.

Release from teaching responsibilities upon retirement from his professorship in 1940 has permitted Dr. Livingston to turn his undiminished energy even more effectively to the improvement of professional organizations of importance to all scientists. His many years of executive work in the American Association for the Advancement of Science and numerous other professional organizations constitute a remarkable record of public-spirited service.

Dr. Livingston has been a pioneer investigator into the primary water relations of plants and inventor of many of the procedures which are standard in that field. Over the past half century he, through his students and through his own personal efforts, has had a remarkably great influence on the progressive development of plant physiology in this country.

The American Society of Plant Physiologists is proud to link the name of Stephen Hales, Father of Plant Physiology, with that of one who has made an equally great contribution. It was with a deep sense of fitness that the Society conferred upon Dr. Burton Edward Livingston the Stephen Hales Award.

The Charles Reid Barnes Award.—Dr. Edwin Cyrus Miller, Professor Emeritus of plant physiology at Kansas State College, was awarded the Charles Reid Barnes Life Membership. Dr. Miller was born December 16, 1878 near Baltimore, Ohio. He received the B.S. degree in 1904 and the A.B. degree in 1906 from the old National Normal University at Lebanon, Ohio. From Yale University he received an A.B. in 1907 and the Ph.D. in 1910. Since that time Dr. Miller has been a member of the staff of Kansas State College and of the Kansas Agricultural Experiment Station. Prior to his retirement last year, Dr. Miller served two years as acting head of the Department of Botany and Plant Pathology.

For forty years Dr. Miller has worked untiringly upon problems of plant physiology, especially those associated with cereal crops. His work has been remarkable for the thoroughness and care with which it has been brought to completion. Plant physiologists are indebted to Dr. Miller for many important contributions to the knowledge of water relations and carbohydrate metabolism, particularly as they apply to the corn plant.

Dr. Miller's book, "Plant Physiology," which appeared first in 1931 with the revised edition in 1938, has provided an outstanding text with extensive bibliographies for advanced classes, graduate students, and research workers in all parts of the world.

Dr. Miller has served as President of the American Society of Plant Physiologists and as a member of the Editorial Board of the journal, PLANT PHYSIOLOGY. He is a member of Sigma Xi, Phi Beta Kappa, Phi Kappa Phi, A.A.A.S. (Fellow), Alpha Zeta, Gamma Sigma Delta, and Farm House Fraternity.

In recognition of Dr. Miller's outstanding accomplishments, the high standards of his professional achievements, and his tenacity in search of answers to technical problems, the American Society of Plant Physiologists presented to him the Charles Reid Barnes Life Membership Award for 1946. With this award go the Society's sincere wishes for many happy years of active and creative work.

Mechanisms of Reactions at Carbon-Carbon Double Bonds.—CHARLES C. PRICE. Interscience Publishers, Inc., 215 Fourth Avenue, New York 3, New York. 120 pages. $2.50.

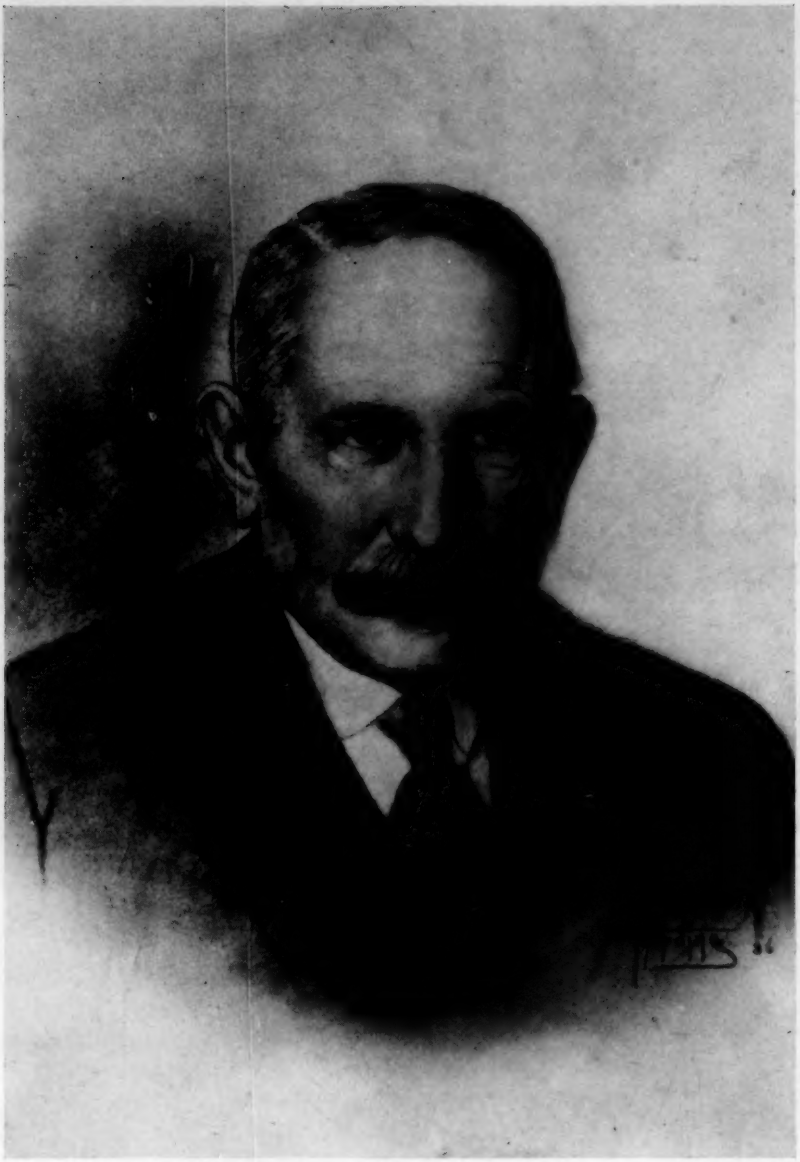
Manuring of Cotton in India.—V. G. PANSE. Indian Central Cotton Committee, Bombay, India. 63 pages. $1.50.

This book is a report of the results of cotton manurial trials carried out in India over a period of years with suggestions for future experiments. Panse, Statistician, Cotton Genetics Research Scheme, Indore, has presented the material in a logical way, including figures and tabulated data.

Books and World Recovery.—The desperate and continued need for American publications to serve as tools of physical and intellectual reconstruction abroad has been made vividly apparent by appeals from scholars in many lands. The American Book Center for War Devasted Libraries has been urged to continue meeting this need at least through 1947. The Book Center is therefore making a renewed appeal for American books and periodicals—for *technical and scholarly books and periodicals in all fields* and particularly for *publications of the past ten years*. We shall especially welcome complete or incomplete files of PLANT PHYSIOLOGY.

The generous support which has been given to the Book Center has made it possible to ship more than 700,000 volumes abroad in the past year. It is hoped to double this amount before the Book Center closes. The books and periodicals which your personal or institutional library can spare are urgently needed and will help in the reconstruction which must preface world understanding and peace.

Ship your contributions to the American Book Center, c/o The Library of Congress, Washington 25, D. C., freight prepaid, or write to the Center for further information.



FREDERICK FROST BLACKMAN

JULY 25, 1866–JANUARY 30, 1947

IN MEMORIAM

FREDERICK FROST BLACKMAN

JULY 25, 1866–JANUARY 30, 1947

FREDERICK FROST BLACKMAN, M.A., D.Sc. (London), F.R.S., Fellow of St. John's College, Cambridge, and formerly Reader in Botany in the University of Cambridge, was born on July 25, 1866, and died at his home in Cambridge, England, on January 30, 1947, in his 81st year.

The name of F. F. BLACKMAN has long been familiar to every student of botany as one of the most distinguished plant physiologists of his time and he is assured of a permanent place in the history of that branch of botanical science to which he devoted his life and to which he, and the school of thought he founded and led, have contributed so much during the past half century or so. Although F. F. BLACKMAN spent the whole of his scientific career at Cambridge (entering as an undergraduate in 1887, appointed Demonstrator in Botany in 1891, and later serving as Lecturer and Reader from 1904 to 1936) his influence so permeates the development of plant physiology in his time that his story belongs in a measure to plant physiologists everywhere. He received the Fellowship of the Royal Society of London in 1906, was President of Section K (Botany) of the British Association in 1908, received other honors and distinctions including a Royal Medal of the Royal Society in 1926, and was President of the Section of Plant Physiology at the International Botanical Congress of 1930. Recognizing this, the American Society of Plant Physiologists elected him their first foreign Corresponding Member in 1932, and in 1934 awarded to him the Charles Reed Barnes Life Membership. Thus while Plant Physiologists everywhere take note of the passing of a great pioneer of their science, the AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS is proud that he may be counted among its most distinguished members.

To appraise the work and influence of F. F. BLACKMAN one must look beyond the papers which bear his name, important as these were and are. His great contribution must be seen in perspective against the unfolding history of botany in his time. The strangely intermittent milestones in the early history of plant physiology in Britain are associated with particular names, familiar to every student, as those of NEHEMIAH GREW, STEPHEN HALES, JOSEPH PRIESTLEY, T. A. KNIGHT, and FRANCIS DARWIN. F. F. BLACKMAN, however, did something more over and above the discoveries identified with his name for he created the first school of plant physiology in England in the modern tradition. The significance of the life and work of BLACKMAN lies in the fact that he stimulated in England a new outlook on plant science as he led the way in applying quantitative experimental methods to the investigation of complex physiologeal proeesses and in for-

mulating interpretations through the application of physico-chemical reasoning.

In a measure BLACKMAN was the product of his time; his greatness lies in the fact that he responded to its challenge. The impetus of the great renaissance of biology as a practical laboratory science under Huxley at South Kensington in the 70's had been carried over into botany by THISELTON-DYER and by S. H. VINES. It is perhaps hard for contemporary teachers to appreciate that even in a country with such an ancient academic tradition as England, practical tuition in botany in the modern sense was unknown prior to the 70's, and plant physiology as a practical laboratory science literally did not exist. Drawing much of his stimulus from the great German physiologist SACHS, VINES was the first advocate of the new practical and experimental outlook on botany at Cambridge and in 1883 he was elected to the newly created post of Reader in Botany. VINES also compiled the first distinctively English textbook on the Physiology of Plants and seemed destined to found at Cambridge the School of Plant Physiology which did not come fully into being till later, for VINES left Cambridge in 1888 to become Professor of Botany at Oxford. The Readership as well as the fostering of the first practical teaching of Plant Physiology in the Botany School at Cambridge then devolved upon FRANCIS DARWIN. In the year 1881, as DR. F. F. BLACKMAN is quoted by F. O. BOWER as saying, "Plant Physiology may be said to have crystallized out in its pure form from the general solution of mixed botanical knowledge," for in ths year the first edition of PFEFFER's textbook appeared. This was the plant physiological scene which awaited F. F. BLACKMAN. He was appointed Demonstrator under FRANCIS DARWIN in 1891 and later succeeded him to the Readership, a post he held until 1936 when he was in turn succeeded by one of his own colleagues and students, DR. G. E. BRIGGS.

Thus the life and active career of F. F. BLACKMAN spans almost the entire modern period of plant physiology in England. Due to his example and the prestige enjoyed by the school which grew up around him, Plant Physiology in England emerged as a branch of botanical thought and discipline worthy to take its place beside the more ancient and firmly established scientific traditions. We shall probably not see his like again for the historical setting which presented him with his opportunity and its challenge will not again return. For the high standards of scholarship which BLACKMAN maintained during this formative period, and the deliberate, calm judgment which pervaded his written works, the science owes him a deep debt of gratitude. Increasingly as the years passed, and until 1936, graduates and graduate students came to be influenced by his teachings, many of them destined to attain eminence and to occupy Botanical Chairs and official positions in England, the Dominions, and in the Empire. Thus it may be said that, from the turn of the century, F. F. BLACKMAN, as no other man, influenced the trend of the subject in the English speaking world.

BLACKMAN leaves no great textbook on the pattern of PFEFFER or SACHS. The actual physiological papers which bear his name are relatively few for so long and so important a career. Plant physiologists are likely to forget that in his earlier years BLACKMAN wrote with authority on the classification and phylogeny of the algae and this interest in algae overlapped the absorbing interest of his career in a discussion of "chromatic adaptation." However, two main series of papers reveal his physiological work and his influence even when he was not avowedly an author. The first of these series began with his own pioneer work and under the series title "Experimental Researches on Vegetable Assimilation and Respiration" appeared in twenty-one successive parts from 1895 to 1933. The other, entitled "Analytical Studies in Plant Respiration," appeared in seven successive parts from 1928 to 1937. These two series go far to tell the story of the Cambridge School of Plant Physiology under F. F. BLACKMAN and, insofar as he leaves a written legacy, we find it here. However, BLACKMAN was always deliberate in his writing and it is common knowledge in Cambridge and in England that much original work done at Cambridge during his period of tenure never reached the stage of publication. This loss to the larger community of plant physiologists can only be offset by the calm, matured judgment that pervades all the papers prepared under the influence of BLACKMAN'S mind.

Reading his papers one is struck with the clarity of his formulations and his ability, where necessary, to rise above the tedious detail out of which great conclusions must often arise. Withal he maintained a dignity of style and language often lacking in our modern reporting of experimental findings and theoretical conclusions. In rereading these early works one comes unexpectedly across occasional touches which seem to carry down the years a breath of the Victorian and Edwardian England in which BLACKMAN moved and worked. His first published papers, communicated to the Royal Society of London by FRANCIS DARWIN in 1884, were really extended notes anticipating by a year in the Proceedings fuller presentations in the Philosophical Transactions. These notes and papers described "A New Method for Investigating the Carbonic Acid Exchange of Plants" and its application to an investigation "On the Path of Gaseous Exchanges between Aerial Leaves and the Atmosphere."

In this first work the method was one which was to be repeated several times as the long series unfolded. First a careful elaboration of a new technical procedure, with painstaking attention to detail, the technique was thoroughly evaluated and carefully described followed by the presentation, in an accompanying paper, of the mature investigation as it was finally carried out. For its time BLACKMAN'S first apparatus was unusually elaborate, but he disarmingly states that: "Simplification of technique by complication of apparatus has been the guiding principle and the result is that, although the whole consists of at least eight separate pieces of apparatus, many of them being further in duplicate and all connected together by a

plexus of tubes, yet the working is so automatically arranged that the operator, beyond reading the burettes and occasionally working a finger bellows, has nothing to do but turn stopcocks."

BLACKMAN showed how by his new technique he could determine the carbon dioxde absorbed or released by a small area of a leaf surface or by a single seed and how he used the method to test whether gas exchange occurred through stomata or through the cuticle. Quantitative data were critically marshalled to show that both CO_2 evolved in the dark and assimilated in the light passed predominantly through the stomata. The cuticular theory had been advocated by BOUSSINGAULT who found that "leaves of *Nerium oleander* assimilated less when the upper astomatiferous surface had been coated with an unguent than when the lower stomatiferous surface had been so coated." BLACKMAN showed conclusively that BOUSSINGAULT'S conclusion flowed from his use of such high concentrations of CO_2 (30%) that when this penetrated freely through the open stomata it inhibited assimilation whereas in the leaf with stomata almost completely blocked, the internal concentraton still allowed assimilation to proceed.

The two subsequent papers in the series published in 1904–1905 recorded work of MISS MATTHAEI on the effect of temperature and illumination on the assimilation of carbon dioxide by leaves of cherry laurel (*Prunus laurocerasus*) and Jerusalem artichoke (*Helianthus tuberosus*). Here the emphasis was upon the interacton of the various factors which affect photosynthesis and upon the fact that under conditions of natural illumination it is the carbon dioxide concentration of the atmosphere which controls the rate of assimilation. Doubtless this phase of the work prompted BLACKMAN'S most famous dictum, enunciated as the Principle of Limiting Factors in 1905 in the Annals of Botany (p. 289), in the following stately passage: "When a process is conditioned as to its rapidity by a number of separate factors the rate of the process is limited by the pace of the slowest factor"; and again, "When the magnitude of a function is limited by one of a set of possible factors, increase of that factor, and of that one alone, will be found to bring about an increase of the magnitude of the function." It matters little whether or not later investigators believe that the principle holds as rigidly as originally conceived. The importance of the principle so simply enunciated lies in the guidance it gave to the analysis and interpretation of experimental data obtained on systems upon which two or more variables interact.

It would be tedious to recapitulate the familiar trend of these researches. The series included papers by THODAY; with A. M. SMITH on photosynthesis in submerged aquatics; by BRIGGS, on the development of photosynthesis in seedlings (a subject studied earlier in BLACKMAN'S laboratory with reference to etiolated seedlings by MISS IRVING); by WILMOTT, who elaborated and improved upon the familiar bubble counting technique with Elodea; by MASKELL, who investigated diurnal rhythm in the assimilation of leaves and made a critical study of assimilation in relation to stomatal opening;

by JAMES, who reinvestigated the dependence of photosynthesis upon carbon dioxide supply using submerged waterplants; and again by BRIGGS, upon the energetic efficiency of photosynthesis in green plants. BLACKMAN'S recognition of slowly progressing thermal or "dark" reactions in photosynthesis which at high light intensity or high carbon dioxide concentrations cause the rate of the overall process to respond to temperature as do chemical reactions is acknowledged by the now general use of the term "Blackman Reaction" to describe this aspect of carbon assimilation.

Analytical studies in plant respiration opened with three papers; two in collaboration or association wth PARIJA and one by F. F. BLACKMAN himself. The new series devoted a great deal of attention to the respiratory behavior of whole, stored apples and, later, of potatoes. The staple technique was again the determination of respired carbon dioxide. The time drifts in the respiratory behavior of stored apples were revealed as well as the changes which ensue when apples are transferred from air to oxygen or nitrogen. On this and similar evidence BLACKMAN formulated a general scheme of plant respiration and enunciated his concept of "Oxidative Anabolism." This was a device to explain the relation between the carbon dioxide evolved in air and in nitrogen. Accepting that the path of carbohydrate breakdown is probably common to both aerobic and anaerobic respiration (at least as far as three carbon intermediates), BLACKMAN postulated that in air part of these intermediates are metabolized to carbon dioxide and water and part resynthesized to products which become reavailable for respiration. In nitrogen, on the contrary, the whole of the three carbon intermediates are utilized to form the products of fermentation. In this concept BLACKMAN followed, or was influenced by, the similar views expressed by MEYERHOF for muscle and yeast, though it must be confessed that the supporting body of biochemical evidence for the systems with which he was concerned was not strong. BLACKMAN'S concept of "Oxidative Anabolism," like the MEYERHOF view, has not passed unchallenged and in particular its generality has been called into question. However, this brings BLACKMAN'S contributions close to the more controversial fields of the present day. The practical science he did so much to foster has moved far from the days when exact measurements of overall gas exchange had yet to be made in order that the problems and processes of plant respiration and photosynthesis could be visualized. Other hands and minds must now thread the way through the often bewildering maze[1] of enzymes and substrates, coenzymes and cofactors, phosphorylations and dephosphorylations, and the sequence of interlocking cyclical processes which seem necessary to explain the biochemistry of these vital processes. F. F. BLACKMAN cleared the way for this task; it will be the simpler because he built so surely.

But what manner of man was F. F. BLACKMAN? Only those who enjoyed close contact with him are entitled to speak here though it is evi-

[1] What Blackman referred to in 1905 as "a congeries of enzymes, a colloidal honeycomb of katalytic agents. . . ."

dent that only a mind of great strength and a rare spirit could have exerted so profound an influence over the long succession of students, collaborators, and colleagues. Perhaps we may be permitted to quote from one who knew him and who writes: "He lived a full and balanced life; his devotion to science did not prevent him from cultivating other interests which included music, architecture, and painting. To problems in all spheres BLACKMAN brought a calm and dispassionate mind and a habit of careful and patient discrimination. There was an unobtrusive strength in his personality which seemed to derive from the blending of wisdom, subtlety of perception and gentleness.

"BLACKMAN was a great teacher as well as a gifted investigator and he linked closely together the two functions of teacher and research. His advanced lectures were based largely upon experimental results obtained in his own school and many of these were (and still remain) unpublished. Each lecture was a perfect presentation in which BLACKMAN succeeded in imparting to his hearers a point of view as well as an ordered array of facts illustrating a particular theme. To attend his lectures was a privilege never to be forgotten and an experience which formed an important part of the discipline of the school of research which grew up under his inspiration."

The AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS therefore joins with botanists and plant physiologists everywhere and pays its tribute to the memory of DR. F. F. BLACKMAN, a great pioneer in the modern experimental approach to the study of plants and plant behavior.

The Editor is indebted to DR. F. C. STEWARD and the Memorial Committee of the Society for the preparation of this memorial biography.